Remarks

Reconsideration of this Application is respectfully requested.

Claims 38-42, 45-49 and 51-57 are pending in the application, with claims 38 and 51 being the independent claims. Claims 1-37, 43, 44 and 50 are cancelled. Claims 38, 47, 48, 49 and 51 have been amended. Support for the amendments to claims 38 and 51 claims may be found throughout the specification. Support for the amendment to claims 47-49 may be found throughout the specification, e.g., in paragraph [0072] on page 23.

Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

Examiner Interview

On June 6, 2007, the Examiner initiated a telephonic interview with Applicants representatives. The Examiner suggested additional amendments to the claims to place the claims in condition for allowance. Applicants thank the Examiner for suggesting the claim amendments and have amended the claims in accordance with the Examiner's suggestions.

The Examiner further requested that Applicants submit copies of Declarations Under 37 C.F.R. § 1.132 by En Li, Ph.D., an inventor, and Kenneth D. Bloch, M.D. that were filed in parent Appl. No. 09/720,086 in support of Applicants' priority claim. Copies of these documents are submitted herewith.

Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw all outstanding objections and rejections.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned directly at (202) 772-8658.

Prompt and favorable consideration of this Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Daniel J. Nevrivy
Agent for Applicants
Registration No. 59,118

Date: June 7, 2007

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 6968
Li *et al.*	Art Unit: 1642
Appl. No. 09/720,086	Examiner: Harris, A. M.
102(e): July 23, 2001	Atty. Docket: 0609.4560002/KRM/DJN
For: ***De Novo* DNA Cytosine Methyltransferase Genes, Polypeptides and Uses Thereof**	

Declaration Under 37 C.F.R. § 1.132 of En Li, Ph.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, En Li, Ph.D., residing at 45 Hinckley Road, Newton, Massachusetts, 02168, declare and state as follows:

1. I am a co-inventor of the above-captioned patent application.

2. I am currently employed by Novartis Institutes for Biomedical Research as Vice President & Global Head, Animal Models of Disease and Epigenetics Program. Prior to my current employment, I was an Associate Professor of Medicine at Harvard Medical School and directed a laboratory in the Cardiovascular Research Center at the Massachusetts General Hospital from January 1993 to April 2003, where I conducted and supervised research in the field of mouse genetics and developmental biology.

3. A current *curriculum vitae* is appended hereto as EXHIBIT A.

4. I have reviewed the above-captioned patent application and the Office Action dated June 6, 2005. I have also reviewed the sequence listing as filed and the sequence listing as amended on July 23, 2001. I have also reviewed the claims of the captioned patent application.

5. I have been informed that the Examiner has not granted priority to the earlier filed patent applications because there is insufficient proof that the coding regions of currently amended **SEQ ID NOS:1** and 2 are the same as those listed in the priority documents, viz., the mouse Dnmt3a and Dnmt3b cDNA clones encoding the coding regions of **SEQ ID NOS:1** and **2**, respectively, that were deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209, USA. Sequences harboring the coding regions of **SEQ ID NOS:** 1 and **2**, respectively, were

deposited with the ATCC on June 16, 1998, and assigned ATCC Deposit Nos. 209933 and 209934, respectively. The deposit date of June 16, 1998 was prior to the filing date of the first provisional application, App. No. 60/090,906, filed June 25, 1998, the benefit of which is claimed. The '906 application includes the sequence information and references the deposits of the sequenced material on page 15, lines 26, through page 16, line 2, of the specification.

6. In November 2004, Applicants had samples withdrawn of the mouse Dnmt3a and Dnmt3b cDNA clones contained within ATCC Deposit Nos. 209933 and 209934, respectively. At the Applicants request, Kenneth D. Bloch, M.D., a faculty member in the Cardiovascular Research Center at the Massachusetts General Hospital and an experienced DNA sequencer, sequenced nucleotides that spanned the coding regions of mouse Dnmt3a and Dnmt3b in the deposited cDNA. A nucleotide alignment that spans the coding regions of sequenced mouse Dnmt3a cDNA clone contained in ATCC Deposit No. 209933 and currently amended **SEQ ID NO:1** is shown in EXHIBIT B. A nucleotide alignment that spans the coding regions of sequenced mouse Dnmt3b cDNA clone contained in ATCC Deposit No. 209934 and currently amended **SEQ ID NO:2** is shown in EXHIBIT C.

7. The amendment to the sequence listing, which was filed on July 23, 2001, corrected six nucleotides in the coding sequence of **SEQ ID NO:1** (see the bolded nucleotides at positions 516, 843, 1036, 1110, 1116 and 1726 in EXHIBIT B) and two nucleotides in the coding sequence of **SEQ ID NO:2** (see the bolded nucleotides at positions 918 and 920 in EXHIBIT C).

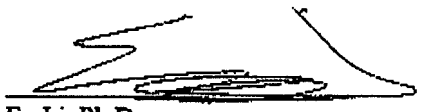
8. The deposited clones recited in ¶¶5 and 6, above (*i.e.*, ATCC Deposit Nos. 209933 and 209934) are the same as the deposited clones recited in the above-captioned application. The coding sequence of ATCC Deposit No. 209933 is currently believed to be the same as the coding sequence of currently amended **SEQ ID NO:1**. The coding sequence of ATCC Deposit No. 209934 is currently believed to be the same as the coding sequence of currently amended **SEQ ID NO:2**.

9. It is well known that sequencing errors are a common problem in Molecular Biology. *See, e.g.*, Peter Richterich, Estimation of Errors in 'Raw' DNA Sequences: A Validation Study, 8 *Genome Research* 251-59 (1998)(EXHIBIT D). I believe that one skilled in the art would have sequenced the deposited material and recognized the sequencing errors in the coding region. I believe that the correct mouse Dnmt3a and Dnmt3b coding sequences are inherent to the ATCC deposited clones, ATCC Deposit Nos. 209933 and 209934, respectively, which were deposited prior to the filing of App. No. 60/090,906, filed June 25, 1998, the benefit of which is claimed.

10. Accordingly, based on the above, I believe that Applicants are entitled to the June 25, 1998 filing date for the coding sequences of mouse Dnmt3a and Dnmt3b contained within ATCC Deposit Nos. 209933 and 209934, respectively.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present patent application or any patent issued thereon.

Respectfully submitted,



En Li, Ph.D.

Date: 11/7/05

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EXHIBIT A

CURRICULUM VITAE

En Li, Ph.D.

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Animal Models of Disease
Novartis Institute for Biomedical Research, Inc.
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Tel: 617-871-7072
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Education:

1984	B.Sc. in Biochemistry. Peking University, Beijing, China
1992	Ph.D. Massachusetts Institute of Technology (Biology) (Advisor: Prof. Rudolf Jaenisch)

Professional Experience:

1993-1996	Principal Investigator, Cardiovascular Research Center, Massachusetts General Hospital Instructor, Department of Medicine, Harvard Medical School
1996-2000	Assistant Professor, Department of Medicine, Harvard Medical School
2000-2003	Associate Professor, Department of Medicine, Harvard Medical School
2001-2003	Guest Professor, Beijing University, Health Science Center
2003-	VP & Global Head, Models of Disease Center, Epigenetics Program Novartis Institute for Biomedical Research.

Review and editorial board

1999-	External Grant Reviewer, Human Frontier Science Program
1999-	External Grant Reviewer, NIH, NICHD
1999-	Member of the Advisory Board. Journal of Biochemistry
2000-	External Grant Reviewer, NIH, NIA
2000-	External Grant Reviewer, NSF
2000-	Mail Reviewer, Wellcome Trust
2003-	Member of the Advisory Board. China Science Reports
2004	External Grant Reviewer, Chinese Natural Science Foundation.
1993-	Ad Hoc Reviewer for the following journals Nature, Science, Cell, Nat Genet, Genes Dev, Trends Genet, Development, Mol Cell Biol, PNAS, Human Mol. Genet., Dev. Biol., Mech. Dev., J. Cell Biol., Dev. Dyn., Gene, Genomics, Nucl Acid Res, Mammalian Genome, etc.

Professional Societies

1990-	American Association for the Advancement of Science, Member

1994- DNA Methylation Society, Member
1999- Ray Wu Society, Member

Invited Presentations (Since 2003-)

2003 Invited speaker, Gordon Research Conference - Cancer Genetics and Epigenetics
2003 Session chair, Keystone Symposium – Chromatin (Big Sky, Montana)
2003 Invited speaker, Annual HUGO meeting at Cancun, Mexico
2003 Invited speaker, Gordon Research Conference – Epigenetics
2004 Invited speaker, the 2nd Annual CDB symposium, Kobe, Japan
2004 Invited speaker, 2nd Weiseenburg Symposium on DNA methylation – an important genetics signals. Weissenburg, Germany
2004 Session Chair, on genomic imprinting. 10th SCBA International Symposiums, Beijing, China
2004 Invited speaker, Genomic imprinting workshop in Montpellier, France
2005 Vice Chair, Gordon Research Conference 'Cancer Genetics and Epigenetics'

Publication:

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EXHIBIT B

EXHIBIT B

Alignment spanning the coding region of mouse Dnmt3a sequence from ATCC Deposit No. 209933 (top) and currently amended SEQ ID NO:1 (bottom)[1]

```
atgccctccagcggccccggggacaccagcagctcctctctggagcgggaggatgatcga
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
atgccctccagcggccccggggacaccagcagctcctctctggagcgggaggatgatcga 276

aaggaaggagaggaacaggaggagaaccgtggcaaggaagagcgccaggagcccagcgcc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
aaggaaggagaggaacaggaggagaaccgtggcaaggaagagcgccaggagcccagcgcc 336

acggcccggaaggtggggaggcctggccggaagcgcaagcacccaccggtggaaagcagt
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
acggcccggaaggtggggaggcctggccggaagcgcaagcacccaccggtggaaagcagt 396

gacacccccaaggacccagcagtgaccaccaagtctcagcccatggcccaggactctggc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gacacccccaaggacccagcagtgaccaccaagtctcagcccatggcccaggactctggc 456

ccctcagatctgctacccaatggagacttggagaagcggagtgaaccccaacctgaggag
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ccctcagatctgctacccaatggagacttggagaagcggagtgaaccccaacctgaggag 516

gggagcccagctgcagggcagaagggtggggccccagctgaaggagagggaactgagacc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gggagcccagctgcagggcagaagggtggggccccagctgaaggagagggaactgagacc 576

ccaccagaagcctccagagctgtggagaatggctgctgtgtgaccaaggaaggccgtgga
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ccaccagaagcctccagagctgtggagaatggctgctgtgtgaccaaggaaggccgtgga 636

gcctctgcaggagagggcaaagaacagaagcagaccaacatcgaatccatgaaaatggag
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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gagtggctggcacgttggaaaagggaggctgagaagaaagccaaggtaattgcagtaatg
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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aatgctgtggaagagaaccaggcctctggagagtctcagaaggtggaggaggccagccct
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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cctgctgtgcagcagcccacggaccctgcttctccgactgtggccaccacccctgagcca
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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```

[1] Bolded nucleotides indicate nucleotides that were amended on July 23, 2001.

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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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tggtggccaggccgaattgtgtcttggtggatgacaggccggagccgagcagctgaaggc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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actcgctgggtcatgtggttcggagatggcaagttctcagtggtgtgtgtggagaagctc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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atgccgctgagctccttctgcagtgcattccaccaggccacctacaacaagcagcccatg
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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cagatgattgaatgggccctcggtggcttccagccctcgggtcctaagggcctggagcca
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```

```
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gacactttctacagtatttcaggtgcctaccacacaggaaaccttgaagaaaaccagttt 3638

ctagaagccgctgttacctcttgtttacagtt
|||||||||||||||||||||||||||||||
ctagaagccgctgttacctcttgtttacagtt 3670
```

EXHIBIT C

EXHIBIT C

Alignment spanning the coding region of mouse Dnmt3b from ATCC Deposit No. 209934 (top) and currently amended SEQ ID NO:2 (bottom)[2]

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gaggagtgcattatcgttaatgggaacttcagtgaccagtcctcagacacgaaggatgct
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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ccctcacccccagtcttggaggcaatctgcacagagccagtctgcacaccagagaccaga
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ccctcacccccagtcttggaggcaatctgcacagagccagtctgcacaccagagaccaga 439

ggccgcaggtcaagctcccggctgtctaagagggaggtctccagccttctgaattacacg
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
caggacatgacaggagatggagacagagatgatgaagtagatgatgggaatggctctgat 559

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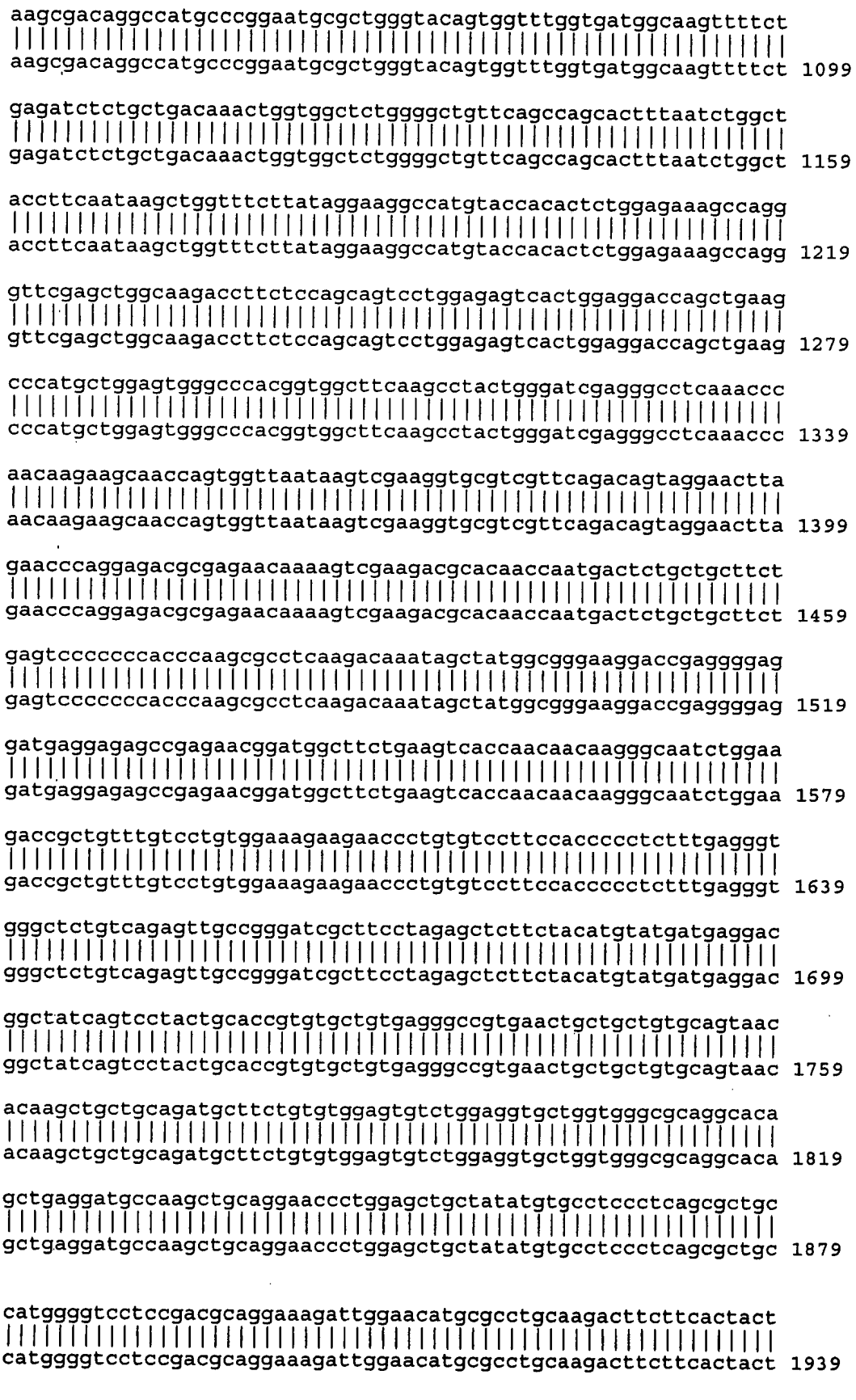
[2] Bolded nucleotides indicate nucleotides that were amended on July 23, 2001.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aagcgacaggccatgcccggaatgcgctgggtacagtggtttggtgatggcaagttttct 1099
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EXHIBIT D

LETTER

Estimation of Errors in "Raw" DNA Sequences: A Validation Study

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As DNA sequencing is performed more and more in a mass-production-like manner, efficient quality control measures become increasingly important for process control, but so also does the ability to compare different methods and projects. One of the fundamental quality measures in sequencing projects is the position-specific error probability at all bases in each individual sequence. Accurate prediction of base-specific error rates from "raw" sequence data would allow immediate quality control as well as benchmarking different methods and projects while avoiding the inefficiencies and time delays associated with resequencing and assessments after "finishing" a sequence. The program PHRED provides base-specific quality scores that are logarythmically related to error probabilities. This study assessed the accuracy of PHRED's error-rate prediction by analyzing sequencing projects from six different large-scale sequencing laboratories. All projects used four-color fluorescent sequencing, but the sequencing methods used varied widely between the different projects. The results indicate that the error-rate predictions such as those given by PHRED can be highly accurate for a large variety of different sequencing methods as well as over a wide range of sequence quality.

In DNA sequencing, knowledge about the accuracy of sequences can be very valuable. For example, different large-scale sequencing projects may produce sequences at similar rates and costs but with significantly different error rates in the final sequence. One major determinant in the final error rate is the accuracy of the "raw" sequence. Knowledge about the frequency and location of errors in the raw sequence data can help to direct "polishing" efforts to the places where additional effort is needed; it also enables the comparison between different sequencing projects without requiring that the same region be sequenced in each project.

Another area where estimates about sequence error rates would be beneficial is technology development. Accurate error estimates at each base would enable "quality benchmarking" between different methods, thus enabling researchers to choose the method that fills their needs for accuracy and throughput best.

Several groups have developed mathematical models to predict the error probability at any given position in raw sequences. Lawrence and Solovyev used linear discriminant analysis to calculate separate probability estimates for insertions, deletions, and mismatches (Lawrence and Solovyev 1994). Ewing and Green (1998) developed the program PHRED, which calculates a quality score at each base. This quality score q is logarithmically linked to the error probability p: $q = -10 \times \log_{10}(p)$ (for a discussion of how quality scores are calculated and what the limitations are, see Ewing et al. (1998). When used in combination with sequence assembly and finishing programs that utilize these error estimates, reliable error probabilities promise to increase the accuracy of consensus sequences and to reduce the efforts required in the finishing phase of sequencing projects (Churchill and Waterman 1992; Bonfield and Staden 1995).

To examine the accuracy of probability estimates made by the program PHRED, we compared the actual and predicted error rates for six different cosmid- or BAC-sized projects that were produced by six different large-scale sequencing centers in the United States. All of these six projects used four-color fluorescent sequencing machines; however, the DNA preparation methods, sequencing enzymes, fluorescent dyes and chemistries, and gel lengths varied significantly between the six groups. Table 1 gives an overview of the sequencing projects analyzed. Table 2 lists the different methods used.

RESULTS

Error Rate Prediction Accuracy for Six Projects

A comparison of actual and predicted error rates for the six projects in this study is shown in Table 3.

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Table 1. Summary of Data Sets

Project	Reads	Aligned bases	Average aligned read length
A	455	416,214	915
B	1277	871,230	682
C	1065	603,655	567
D	834	414,595	497
E	1638	1,149,209	702
F	1885	907,796	482
Total	7154	4,362,699	610

The results indicate that PHRED is very successful in identifying bases with low error probabilities. For example, the 1.28 million bases with quality scores of 4–12 (corresponding to error probabilities between 39.8% and 6.3%) contain a total of 187,926 errors. In contrast, the 1.44 million bases with quality scores between 33 and 42 (corresponding to error probabilities between 0.05% and 0.006%) contain only 237 errors, which translates into a 790-fold lower error rate. The trend toward lower error rates can also be observed for each individual project. In most cases, the actual number of errors is close to the predicted error rate. It is also apparent that the actual error rate is typically lower than the predicted error rate.

Both the high overall accuracy and the tendency to slightly overpredict errors are confirmed by statistical analysis, as shown in Table 4. The correlation between predicted and actual error frequencies is excellent for all projects (Spearman correlation coefficient >0.89, $P < 0.0001$). Averaged over all projects, the actual error rate is 84.5% of the predicted error rate; the slope of the relation between predicted and actual error rates differs slightly between projects and ranges from 76.6% to 88.4%. To put these differences between projects in relation, it is worthwhile remembering that PHRED quality scores cover a wide dynamic range: The maximum quality score of 51 corresponds to a 50,000-fold lower predicted error rate than the minimum quality score of 4. Even the relative difference between successive quality is larger than the relative difference in the slopes; for example, a quality score of 10 corresponds to an error probability of 10%, whereas a score of 9 corresponds to an error probability of 12.6%.

A different way of looking at the relation between the actual and predicted error rates is shown in Figure 1. Here, the error rates as a function of the position within all reads in each of the projects, averaged over 50-base windows, is depicted. For all six projects, the predicted error rates are very close to the actual error rates over the entire length of the sequences. Each project has a characteristic distribution of error rates, which differs from each of the other projects. The minimum error rate differs dramatically between projects. The best projects achieve raw error rates of 0.23%–0.36% in the best region of the sequence read, typically from base 150 to 200. The worst project in the data set had an ~10-fold higher error rate of 2.58%.

Toward the end of sequence reads, the error rates increase and start to exceed 10% between bases 300 and 700. In projects that used mainly short gels (e.g., projects D and F), this increase begins sooner, whereas projects that use longer gels show a markedly longer stretch of low error rates (e.g., projects A and B).

Table 5 summarizes key results for the six projects. The first four projects have similar minimum and average error rates. However, the length of the region where the error rate is below 5% differs significantly, from 403 to 682 bases. The project with the shorter low error rate regions contained larger portions of reads generated on short gels, whereas projects A and B were run exclusively on long gels (ABI373 stretch or ABI377 sequencers). Other factors contributing to differences between the first four projects were differences in sequencing chemistries, production scale, and electrophoresis conditions and machines.

Project E and, in particular, project F, had significantly higher error rates than the first four projects. In projects E and F, every sequence generated for the project had been included in the data set, whereas the other four projects had eliminated some "bad" sequences through manual or auto-

Table 2. Overview of Sequencing Methods Used in the Different Projects

Template DNA	single-stranded M13, double-stranded plasmids
Sequencing enzymes	Sequenase, *Taq*, KlenTaqTR, AmpliTaq FS
Sequencing chemistries	Dyes primer (two different dyes chemistries), dye terminator
Sequencing machines	ABI 373, ABI 373 stretch, ABI 377
Gel length	Only short gels, only long gels, mixes of short and long gels

Table 3. Comparison of Predicted and Actual Error Rates for Six Different Sequencing Projects

Project	Quality score	4–12	13–22	23–32	33–42	43–51
A	aligned bases	119,246	75,293	70,391	144,876	73,234
	expected errors	20,256	2,064	172	37	1
	actual errors	16,784	1,758	127	17	1
B	aligned bases	182,034	137,940	181,998	399,690	140,176
	expected errors	29,953	3,704	410	102	3
	actual errors	26,038	2,536	287	35	0
C	aligned bases	139,345	131,419	151,197	292,070	68,529
	expected errors	22,277	3,411	357	74	2
	actual errors	16,670	1,513	194	26	3
D	aligned bases	103,898	68,995	68,613	153,730	111,752
	expected errors	16,880	1,919	168	38	3
	actual errors	14,495	1,924	146	59	2
E	aligned bases	378,755	217,438	167,968	392,717	144,313
	expected errors	63,947	6,336	418	95	4
	actual errors	55,968	6,516	355	67	5
F	aligned bases	359,809	136,688	98,840	64,035	5,130
	expected errors	66,938	4,079	256	23	0
	actual errors	57,971	3,856	332	33	1
All	aligned bases	1,283,087	767,773	739,007	1,447,118	543,134
	expected errors	220,252	21,513	1,781	370	13
	actual errors	187,926	18,103	1,441	237	12

matic inspection. After eliminating <10% of the worst sequences in project F, the error rates for the remaining sequences were comparable to those of the first four projects. In contrast, project F showed a much more uniform distribution of sequence quality.

The last column in Table 5 shows the average number of bases with an estimated error probability of at most 0.1%, which is equivalent to a quality score of at least 30. The count of such "very high-quality" bases is a good indicator of sequence quality, both for individual sequences and, when aver-

Table 4. Summary of Statistical Analysis Results

Project	Spearman ρ	$P > \|\rho\|$	Slope	*t* ratio	$P > \|t\|$
A	0.9646	<0.0001	0.818	75.1	<0.0001
B	0.9890	<0.0001	0.874	98.2	<0.0001
C	0.9846	<0.0001	0.766	71.6	<0.0001
D[a]	0.8692	<0.0001	0.855	68.3	<0.0001
E	0.9956	<0.0001	0.884	144.3	<0.0001
F	0.9968	<0.0001	0.865	151.6	<0.0001
All	0.9964	<0.0001	0.845	174.5	<0.0001

[a]In project D, the Spearman correlation coefficient ρ was artificially low as only very few bases (10) bases had a quality score of 5, and none of these bases contained an actual error (expected: 3.16 errors). Exclusion of this quality score gave a Spearman correlation coefficient of 0.9786 ($P < 0.0001$). The frequencies in the slope calculations were weighed by the number of bases at any given quality score and, thus, were not sensitive to such small sample distortions (see Methods).

ity analysis and control in large-scale DNA sequencing projects. To analyze how accurate PHRED error estimates are for different quality sequences within the same sequencing project, we subdivided a data set into four quartiles, based on the number of very high-quality bases in each sequence (see Methods). The comparison of actual and predicted error rates is shown in Figure 2.

When measured by the error rate in the best region of a sequence, the data quality in the different quartiles varies >100-fold between the best and the worst 25% of the sequences. The best quartile showed ~0.03% error for >100 bases, whereas the error rate in the worst quartile always exceeded 5%. In quartiles 2 and 3, the predicted error rates match the actual error rates very closely. In the best and worst quartiles, PHRED's accuracy was somewhat lower from base 100 to 500. In the best sequences, PHRED's error estimates were about twofold too high; in the worst sequences, the error estimates were too low, again by a factor of 2. This underprediction of errors can be partially explained by the fact that PHRED gives ambiguous base calls (*N*'s) a quality score of 4, corresponding to an error probability of 39.8%; however, *N*'s will always show up as an actual error. Even in the worst and best quartiles, however, the predicted error rate curves are very similar to the actual error rate curves.

The results shown in Figure 2 also demonstrate that the count of very high-quality bases, or bases with an estimated error probability of at most 0.1%, can be used effectively to characterize the overall quality of a sequence read. Sorting the sequence reads into quartiles based on the number of very high-quality bases worked well, as shown by the >100-fold difference in the minimum error rate between the first and the fourth quartile.

Other methods to characterize the overall quality of individual reads based on PHRED quality scores can give similar results. For example, counting bases above a minimum quality threshold anywhere in the range of 20–40 gave similar results for most data sets (not shown), and such counts are used by a number of different laboratories as quality measures. Alternatively, the quality values can be converted to error probabilities and averaged to give the predicted error rate for the trace, or summed to give the total predicted number of errors in a trace. However, such averages and totals can sometimes give a misleading picture, as the following example illustrates. Assume that two sequence reads have very similar quality in the alignable part of the read but that one of the two sequences was run much longer and

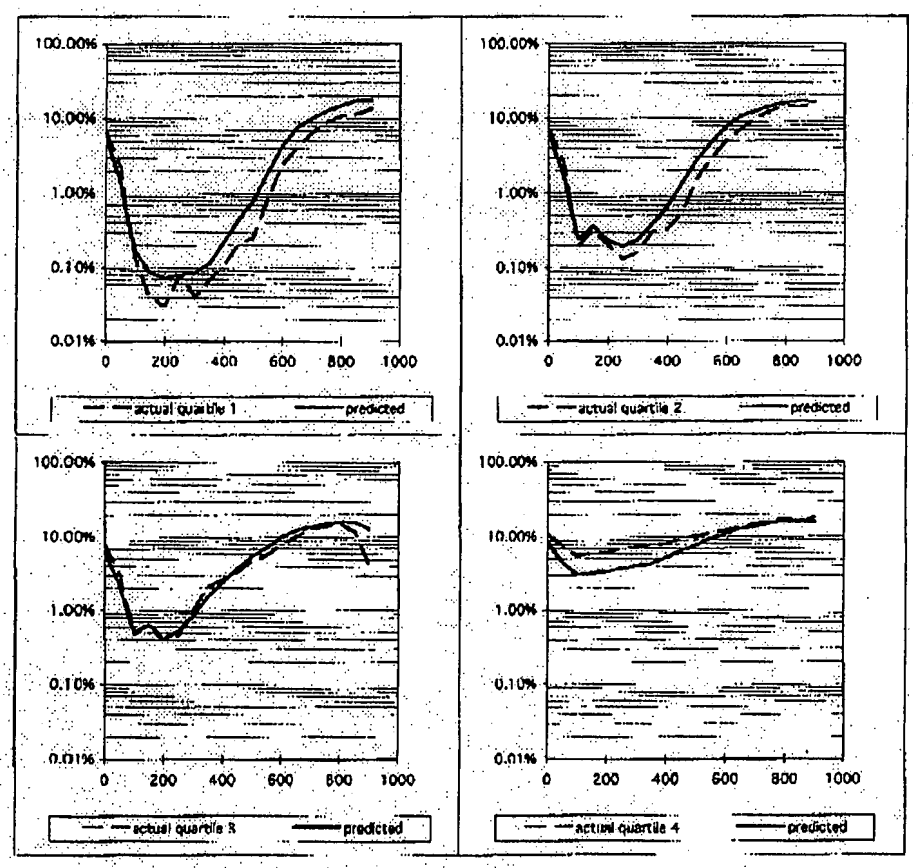

Figure 2 Actual and predicted error rates in different quality subsets of project B. Sequence reads were sorted by the number of bases with a predicted error rate of at most 0.1% (very high-quality bases), and assigned to quartiles, with quartile 1 corresponding to the highest numbers. Actual and predicted error rates for all sequences in each subset were calculated as in Fig. 1. Note that a number of sequence reads that had been rejected because of too low quality were added back to the data set for illustrative purposes, all of which are in quartile 4. These sequences were not included in the data sets used to generate Figs. 1 and 3 and Tables 1 and 3.

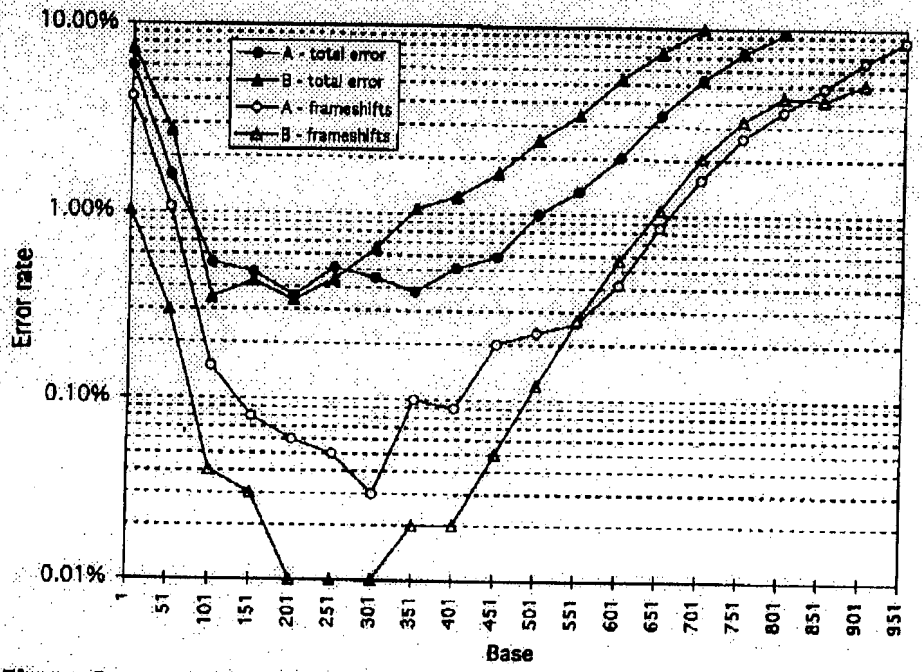

Figure 3 Actual frameshift and total error rates for projects A and B. To calculate frameshift error rates, only insertions and deletions were counted. Mismatch errors, which account for the vast majority of errors after base 150, were included only in the total error count. Note that project B (▲,△) has a slightly similar or slightly higher total error rate compared to project A (●,○) but only about one-third as many insertions and deletions up to base 500. For both projects, the frameshift error rate in the raw data is <1 in 1000 for >300 bases, and ≤1 in 10,000 for >100 bases in project B.

therefore contains a longer unalignable "tail" of very low-quality bases. When calculating the average error rate for these two sequences, the second sequence will have a much higher average error and, therefore, appear to be of lower quality. In contrast, the counts of very high-quality bases for both sequences will be very similar, as the unalignable tails contain few, if any, high-quality bases. Therefore, counts of bases above a high enough quality threshold will give a more robust and clearer picture of trace quality.

Frameshift Error Rates for Different Sequencing Chemistries

Depending on how biologists use DNA sequences, knowledge about total error rates in raw sequences may or may not be sufficient. For example, frameshift errors in coding sequences will generally lead to incorrectly predicted open reading frame, whereas mismatch errors will do so only if the mismatch introduces a stop codon or a new splice site. At the time of this writing, PHRED did not differentiate between mismatch and frameshift errors, but only estimated total error rates. This might occasionally lead to questionable conclusions, as the results shown in Figure 3illustrate.

Figure 3 shows the total actual error rates and the frameshift error rates for two projects, A and B. The total error rates for both projects are similar for up to 350 bases; after 350 bases, project B has a somewhat higher total error rate. However, examining the frameshift error rate gives rise to a different picture: from base 1 to 500, project A has approximately four times as many insertions and deletions as project B. This difference in frameshift error rates can be explained by the sequencing chemistries that were used in the two projects. Project B, with the lower frameshift error rate, used only dye terminator chemistry, which is known to eliminate band spacing artifacts from hairpin structures ("compressions"). Project A, on the other hand, used dye primer chemistry, which is more prone to insertion and deletion errors from mobility artifacts, for most sequencing reactions.

DISCUSSION

As large-scale DNA sequencing has become a more routine and common process, the traditional methods for assessing sequence quality have become unsatisfactory. In projects like single-pass cDNA sequencing, it is not possible to calculate and compare error rates after finishing a sequence, as finishing never takes place. Even when a comparison between raw and finished sequence can be done, the time delay between raw data generation and quality assessment is often large. This delay makes it difficult to improve ongoing projects, and it sometimes makes it impossible to capture problems early on. Some immediate quality feedback can be reached by including known standard sequences for quality control. However, this approach can be costly, and it fails when error profiles differ between standard and unknown sequences.

In contrast to these traditional methods to assess sequence accuracy, direct estimation of error

rates in raw sequence data would enable immediate quality control and feedback. Accurate, base-by-base estimates of error probabilities could also increase the utility of single-pass sequences significantly, allow efficient comparison and optimization of different sequence chemistries, and enable the development of better software tools for sequence assembly and analysis.

The critical question for any error rate prediction tool is how accurate are the error rate estimates, in particular if different sequencing methods and chemistries are used? The results presented herein provide an answer to this question for the program PHRED, as well as clues where further development would be useful. As shown in Tables 3 and 4 and in Figure 1, the agreement between predicted and actual error rates was very good in each of the six different projects analyzed. The observed high level of prediction accuracy in all of these projects is almost astonishing if one takes into account that actual errors are binary (a base is either correct or wrong), whereas predicted error rates are probabilities on a scale from 0.0 to 1.0. The observed tendency to overpredict error rates can be at least partially explained by the "small sample correction" that was used in the derivation of threshold parameters for quality scores (Ewing and Green 1998). For most practical applications, such a somewhat conservative estimation of quality scores is tolerable or even desirable. Overall, the results clearly show that error probabilities given by PHRED accurately describe raw sequence data quality.

In judging the usefulness of predicted error probabilities, it is important to know how differences in sequencing methods will influence the prediction accuracy. For example, the larger variation in peak heights tends to be larger in dye terminator sequencing than in dye primer sequencing, and different sequencing enzymes are known to produce different specific height variation patterns. Any estimation of error probabilities that takes the peculiarities of a specific sequencing chemistry into account would therefore be expected to be less accurate for different chemistries.

The projects included in this study were specifically chosen to provide an initial answer to the question of how generally useful PHRED quality scores are. These projects represent the vast majority of different multicolor fluorescent sequencing methods used in the last 3 years: different template DNAs and DNA preparation methods, different enzymes, gel lengths, run conditions, and different fluorescent dyes. The data also include a considerable spread in data quality, both between projects and within individual projects. None of the projects analyzed here were included in PHRED's training set, and just one of the six laboratories that contributed data to this study also contributed data to the training data sets. One of the projects in this study consisted entirely of dye terminator sequences, which presented only a small fraction of the sequences in the test data set. Another project exclusively used a set of fluorescent dyes different from those used in the training sets. Each project differed from the other projects in this study in at least one, and typically many, experimental aspects like template preparation, sequencing enzymes, gel run conditions, and so forth. Despite these differences, the accuracy of error rate predictions was very similar for all projects.

Our results justify some optimism about the accuracy of PHRED quality scores for minor changes in sequencing technology, for example, sequences generated by new enzymes and fluorescent dyes. Initial studies showed that PHRED quality scores were also accurate for sequences produced by multiplex sequencing with radioactive detection (P. Richterich, unpubl.). However, we also observed two effects that can invalidate PHRED quality scores during these studies. First, sequences generated by chemical sequencing gave too low quality scores at mixed (A + G) reactions. Because secondary peak height is one of the parameters used in the error rate predictions, this is not surprising. Another potential source of error is high-frequency noise in the trace data. With such data, PHRED occasionally underestimated the band spacing by a factor of 2 or more, which resulted in incorrect base calls and quality scores. By applying simple smoothing algorithms to data with high-frequency noise, these problems could typically be resolved. Similar steps may be necessary to obtain accurate PHRED quality scores on data that have been generated by different sequencing instruments or preprocessed by different software.

Accurate quality scores can have a major impact on how sequences are used downstream from the sequence production process. In traditional sequencing projects where the goal is complete coverage at a final error rate below (e.g.) 1 in 10,000, the accuracy goals can be reached with single sequence reads as long as the quality scores are at least 40 (however, other potential problems like clone instability may make higher coverage advisable). Interesting questions arise as to how individual read quality contributes to project quality, or the error rate of the "final" sequence. Under the assumption that errors between different sequence reads are

completely independent, one could argue that two reads with a quality score of 20 (error probability of 1 in 100) are just as valuable as one sequence with a quality score of 40 (error probability of 1 in 10,000). However, although a single sequence stretch with quality levels above 40 would give a final sequence with an error rate of <1 in 10,000, assembling a consensus from two sequences with quality scores of 20 (1% error rate) could lead to one of two results: If the errors were completely random, the consensus sequence would be ambiguous at 2% of all locations; if the errors were completely localized, for example, because of reproducible compressions, the consensus sequence would have one "hidden" error every 100 bases. Typically, consensus sequences derived from low-quality sequences will have both kinds of problematic regions. Increased coverage can rapidly eliminate the random errors; however, increased coverage does not resolve errors from systematic sources. Manual examination of such problem areas is generally required; such "contig editing," however, tends to be time consuming, requires highly trained personnel, is an obstacle toward complete automation of DNA sequencing, and sometimes fails to eliminate all errors. This leads to the somewhat counterintuitive conclusion that the practical value of increasing sequence quality can be even higher than indicated by the quality scores: One sequence of average quality above 40 can be "worth" more than two sequences of average quality 20.

Another application of DNA sequencing where high quality can be of disproportionately high value is the search for mutations in genomic DNA. In low quality sequences, secondary peaks and low resolution often complicate the identification of heterozygous mutations. In regions of higher sequence quality, such secondary peaks are smaller or absent and peaks are better resolved. Therefore, both false-positive and false-negative errors can be significantly reduced in high-quality regions. Tools like PHRED, which can accurately measure sequence quality from trace data, can be of twofold value for mutation detection. First, base-specific quality scores can allow optimization of sequencing methods and strategies for mutation detection. Second, the quality scores can be used to evaluate the usefulness of individual sequence reads for mutation detection (e.g., by discarding reads below minimum thresholds), and they can guide software that automatically detects mutations.

The ability to predict error rates in a highly accurate fashion is likely to have a major impact in applications like those described above. PHRED is the first widely used program that accurately predicts base-specific error probabilities. However, the algorithm for determining quality values has been described (Ewing and Green 1998), and it should be straightforward to implement similar quality values in other base-calling programs. Furthermore, an extension of the approach developed by Ewing and Green should be possible. For example, differentiation between mismatch and frameshift errors would enable better comparisons of sequencing methods with similar total error rates but different frameshift error rates. Several groups have described efforts to calculate separate probabilities (or "confidence assessments") for mismatch errors and frameshift errors (Lawrence and Solovyev 1994; Berno 1996). Their results demonstrated that different approaches to error type characterization are feasible and promising. Implementation of such error type predictions in other programs similar to the way PHRED uses quality scores would enable better method assessments, benchmarking, and production quality control, and could have a significant impact on downstream uses of DNA sequence information.

METHODS

Data Sets

For one project, sequence raw data in the form of ABI trace files were downloaded from a public FTP site. Sequence data for the five other projects were kindly provided by five different large-scale sequencing groups. Table 1 gives a summary of the six projects, and Table 2 gives an overview of the different sequencing methods used in the projects. The projects differed in the amount of prescreening of data that had been done, reflecting different approaches to quality control in different laboratories. In two projects (B and C), different software programs had been used to identify and eliminate low-quality sequences. One project (F) included all data files generated, whereas the other three projects had excluded "failed lanes."

Comparison of Actual and Predicted Error Rates

The sequences for all traces in each project were recalled using the program PHRED (v. 961028). Next, sequences in each project were assembled with PHRAP (P. Green, unpubl.). Slightly different methods were chosen for the statistical and graphical evaluation of the error rate prediction accuracy. In the statistical evaluation, only the longest contig produced by PHRAP was considered. The tables of aligned bases and observed discrepancy counts for

each quality score were taken from the PHRAP output and analyzed as follows. The expected number of discrepancies (E) at each quality score (q) was calculated by multiplying the number of aligned bases (N) with the error probability corresponding to the quality score: $E = N\ 10^{-q/10}$. The Spearman ranking coefficients were calculated by comparing the expected and observed error frequencies. To obtain the quantitative relation between the expected and observed error rates over the entire range, a least-squares fit between the observed and expected rates was performed, with the intercept set to zero and the number of aligned bases at each quality score used as weights.

For a graphical comparison of estimated and actual error rates in 50-bp windows, the following steps were taken. For two of the projects, the consensus sequence was retrieved from public databases. For the four other projects, the DNA sequence and quality information were used by the program PHRAP to assemble consensus sequences for each of the projects. The individual reads were aligned to the consensus sequences of the longest contig, using the program CROSS_MATCH (P. Green, unpubl.), after removing single-coverage regions from the ends of the consensus sequence. CROSS_MATCH uses an implementation of the Smith Waterman algorithm to generate alignments that typically do not include the ends of sequences, where disagreements are commonly due to vector sequence or low quality sequence.

The quality files generated by PHRED and the alignment summaries generated by CROSS_MATCH were then analyzed as follows. First, the region of each query sequence that had been aligned by CROSS_MATCH was determined. Next, the actual and predicted error rates for the entire aligned part of each individual sequence was calculated. In addition, the average actual and predicted error rates for all alignable sequences together were calculated for windows of 50 bases in length. To calculate the predicted error rate, the quality scores q determined by PHRED at each base were converted to error probabilities as described above (Ewing and Green 1998).

Subdividing Data Into Subsets Based on Data Quality

To examine the accuracy of PHRED quality scores for data subsets of different quality within a project, the following approach was taken. For all sequence reads in project B, the number of bases with a quality score of at least 30 in each sequence was determined (bases with quality scores of at least 30 were called very high-quality bases, or VHQ bases). Sequences were sorted in descending order based on the number of very high-quality bases, and divided into four quartiles. Accordingly, quartile 1 contained 25% of sequences with the highest number of very high-quality bases, and quartile 4 contained the "worst" sequences. To illustrate the prediction accuracy in data with relatively high error rates, sequences from project B that had been "discarded" because they had not met the minimum quality criteria were added back to the data set. The sequences in each quartile were compared to the consensus sequences that had been generated using the entire data set, as described above for the graphical comparison.

Determining Actual Frameshift Error Rates

The calculation of actual frameshift error rates in the raw sequence data was performed using CROSS_MATCH, similar to the procedure described above for total error rates, except that only insertion and deletion errors were counted. Because PHRED does not give separate frameshift error estimates, a comparison of predicted and actual frameshift errors is not possible.

ACKNOWLEDGMENTS

I thank the participating laboratories for contributing their data, Dr. Josée Dupuis for help with the statistical analysis, and Dr. Phil Green for helpful discussions.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Li *et al.*	Confirmation No.: 6968
Appl. No. 09/720,086	Art Unit: 1642
102(e): July 23, 2001	Examiner: Harris, A. M.
For: ***De Novo* DNA Cytosine Methyltransferase Genes, Polypeptides and Uses Thereof**	Atty. Docket: 0609.4560002/KRM/DJN

Declaration Under 37 C.F.R. § 1.132 of Kenneth D. Bloch, M.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, Kenneth D. Bloch, M.D., residing at 80 Park Street, Apt. 32, Brookline, Massachusetts, 02446, declare and state as follows:

1. I am currently employed by Massachusetts General Hospital in the Cardiovascular Research Center where I conduct and supervise research in the field of molecular cardiology. I worked with Dr. En Li, co-inventor of the captioned application, in the Cardiovascular Research Center for 10 years until 2003 when Dr. Li moved to Novartis. I am also an Associate Professor of Anesthesiology and Medicine at Harvard Medical School and have extensive experience in molecular biology and DNA cloning and sequencing.

2. A current *curriculum vitae* is appended hereto as EXHIBIT A.

3. I have been informed that human DNMT3A cDNA clone, represented in the captioned patent application as **SEQ ID NO:3**, was deposited with the ATCC on July 10, 1998, and assigned ATCC Deposit No. 98809. I have also been informed that the deposit date of July 10, 1998 was prior to the filing date of the second provisional application, App. No. 60/093,993, filed July 24, 1998, the benefit of which is claimed. Finally, I have been informed that the '993 application includes the sequence information and references the deposit of the sequenced material on page 16, lines 1-2, of the specification.

4. In November 2004, Applicants had samples withdrawn of the human DNMT3A cDNA clone contained within ATCC Deposit No. 98809. At the Applicants request, I have sequenced the nucleotides that span the coding region of the deposited human DNMT3A cDNA clone contained in ATCC Deposit No. 98809. A nucleotide alignment that spans the coding regions of the sequenced human DNMT3A cDNA clone contained in

ATCC Deposit No. 98809 and currently amended **SEQ ID NO:3** is shown in Fig. 1 of EXHIBIT B.

5. The amendment to the sequence listing, which was filed on July 23, 2001, corrected six nucleotide errors in the coding sequence of **SEQ ID NO:3** (see bolded nucleotides at nucleotide positions 940, 1476, 1479, 1570, 2024 and 2119 of amended **SEQ ID NO:3** in Fig. 1 of EXHIBIT B). The amendment also deleted original nucleotides 1-123 of **SEQ ID NO:3**, which does not include any DNMT3A coding sequence.

6. The deposited clone recited in ¶¶4 and 5, above (*i.e.*, ATCC Deposit No. 98809) is the same as the deposited clone recited in the above-captioned application. The six nucleotides in the coding region of **SEQ ID NO:3** that were corrected by the amendment of July 23, 2001 correspond to the sequence contained in ATCC Deposit No. 98809. It is well known that sequencing errors are a common problem in Molecular Biology. Peter Richterich, Estimation of Errors in 'Raw' DNA Sequences: A Validation Study, 8 *Genome Research* 251-59 (1998)(EXHIBIT C). I believe that one skilled in the art would have sequenced the deposited material and recognized the sequencing errors.

7. My sequencing of ATCC Deposit No. 98809 also revealed that nucleotides 539-584 within the coding region of amended **SEQ ID NO:3** are deleted in the deposited cDNA. The deletion causes a frame shift in the coding region of the deposited cDNA and predicts a truncated protein of 145 amino acids. An amino acid alignment of the predicted amino acid sequence encoded by the human DNMT3A cDNA clone contained in ATCC Deposit No. 98809 and the predicted amino acid sequence encoded by amended **SEQ ID NO:3** is shown in Fig. 2 of EXHIBIT B. The bolded sequence in Fig. 2 corresponds to the predicted encoded amino acid sequence downstream of the nucleotide deletion in ATCC Deposit No.98809 and represents a point of divergence compared with the predicted amino acids encoded by currently amended **SEQ ID NO:3**.

8. Currently amended **SEQ ID NO:3** and **SEQ ID NO:3** as originally filed in U.S. Appl. Nos. 60/090,906 and 60/093,993, to which priority is claimed, do not harbor the deletion, and encode a protein having 912 amino acids that is homologous to mouse Dnmt3a.

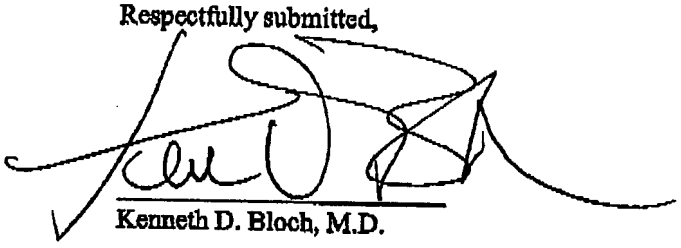
9. Like DNA sequence errors, it is known that errors in DNA cloning may occur in molecular biology. Deletion errors may occur and may be caused by, *inter alia*, inadvertent digestion of DNA by restriction endonucleases or exonucleases, or by recombination events during propagation of the DNA in bacterial hosts. The deletion at nucleotides 539-584 in **SEQ ID NO:3** present in ATCC Deposit No. 98809 is an obvious error. I believe that one skilled in the art would have sequenced the deposited material and recognized the deletion as an error. My belief is based upon the following: First, the deletion found in ATCC Deposit No. 98809 is not present in **SEQ ID NO:3** as originally filed or as amended. Second, the deletion is not present in the DNA sequence of the closely related mouse homolog, SEQ ID NO:1. Third, the deletion causes a frame shift in the reading frame of **SEQ ID NO:3** and predicts a truncated protein product compared with that

encoded by **SEQ ID NO:3** as originally filed and as amended. Fourth, the amino acids encoded by the nucleotide sequence downstream of the deletion bear no similarity to the amino acids encoded by **SEQ ID NO:3** or the mouse homolog of Dnmt3a, encoded by SEQ ID NO:1. Finally, an examination of the sequence reveals two large open reading frames (ORF) in the sequence in different frames. See Fig. 3 of EXHIBIT B. The ORFs correspond to the amino acid residues of DNMT3A upstream and downstream of the deletion. The presence of two large ORFs in different frames indicates a possible frameshifting sequence error or deletion. All of these factors indicate that the deletion present in ATCC Deposit No. 98809 is an error, and would be recognized as such by a person of ordinary skill in the art.

10. It is my belief that the combination of ATCC Deposit No. 98809, which discloses the six nucleotides in the coding region of **SEQ ID NO:3** amended on July 23, 2001, in combination with **SEQ ID NO:3** as originally filed in U.S. Appl. Nos. 60/090,906 and 60/093,993, which disclose nucleotides 539-584 of amended **SEQ ID NO:3**, clearly conveys to someone skilled in the art the entire nucleotide sequence of amended **SEQ ID NO:3.**

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present patent application or any patent issued thereon.

Respectfully submitted,



Kenneth D. Bloch, M.D.

Date: 11/7/2005

EXHIBIT A

CURRICULUM VITAE

PART I: General Information

DATE PREPARED: July 5, 2005

Name: Kenneth D. Bloch

Office Address: Cardiovascular Research Center
Massachusetts General Hospital
149 13th Street (149-4201)
Charlestown, MA 02129
(617) 724-9540

Home Address: 80 Park Street, Apartment 32, Brookline, MA 02446

E-Mail: kdbloch@partners.org **FAX:** (617) 726-5806

Place of Birth: New York, NY

Education:

1978	Sc.B.	Brown University (Biomedicine)
1981	M.D.	Brown University

Postdoctoral Training:

Internship and Residencies:

1981-1984	Internal Medicine, Massachusetts General Hospital

Fellowships:

1981-1984	Clinical Fellow in Medicine, Harvard Medical School
1984-1987	Research Fellow in Genetics, Harvard Medical School
1987-1989	Clinical and Research Fellow in Medicine, Harvard Medical School

Licensures and Certification:

1984-	Commonwealth of Massachusetts, Registered Physician,
1985	Diplomate, American Board of Internal Medicine
1989	Diplomate, Subspecialty Board of Cardiovascular Diseases, American Board of Internal Medicine

Academic Appointments:

1989-1990	Instructor in Medicine, Harvard Medical School
1990-1997	Assistant Professor of Medicine, Harvard Medical School

1997-	Associate Professor of Medicine, Harvard Medical School
2005-	Associate Professor of Anaesthesia, Harvard Medical School

Hospital or Affiliated Institution Appointments:

1990-1996	Assistant in Medicine, Massachusetts General Hospital
1996-1999	Assistant Physician, Massachusetts General Hospital
1999-2005	Associate Physician, Massachusetts General Hospital
2005-	Physician, Massachusetts General Hospital

Other Professional Positions and Major Visiting Appointments: none

Hospital and Health Care Organization Service Appointments:

1990-	Attending Physician, Cardiology and Medical Services, Massachusetts General Hospital
1991-	Practice Member, Cardiac Unit Associates, Massachusetts General Hospital

Major Administrative Responsibilities:

1989-1992	Principal Investigator, Cardiac Unit, Massachusetts General Hospital
1990-	Associate Program Director, Fellowship Program in Cardiovascular Disease, Massachusetts General Hospital
1992-	Principal Investigator, Cardiovascular Research Center, Massachusetts General Hospital
1993-1997	Preceptor, Training Grant to the Cardiovascular Research Center (PI: Mark C. Fishman), Massachusetts General Hospital
1997-2002	Co-Investigator, Training Grant to the Cardiovascular Research Center (PI: Mark C. Fishman), Massachusetts General Hospital
2002-2004	Interim Director, Cardiovascular Research Center, Massachusetts General Hospital
2002-	Principal Investigator, Training Grant to the Cardiovascular Research Center, Massachusetts General Hospital

Major Committee Assignments:

Hospital:

1991	Member, Selection Committee for the Fellowship Program in Cardiovascular Disease, Massachusetts General Hospital
1992-1996	Member, Subcommittee on Review of Research Proposals, Committee on Research, Massachusetts General Hospital
1997-	Member, Steering Committee coordinating the integration of the clinical cardiology fellowship programs at Brigham and Women's Hospital and Massachusetts General Hospital

Regional:

1991-1994	Member, Research Peer Review Committee, American Heart Association, Massachusetts Affiliate, Inc.
2002	Member, Northeast Peer Review Study Group for Lipids, Thrombosis & Vascular Wall Biology, American Heart Association

National:

1992-1995	Member, Molecular Signaling Research Study Committee, American Heart Association, National Center
1996-1997	Member, Lung Biology and Pathology Study Section, National Heart Lung and Blood Institute
2000	Member-At-Large, Executive Committee of the Council on Cardiopulmonary and Critical Care, American Heart Association, National Center
2000-2002	Member, Program Committee, Council on Cardiopulmonary and Critical Care, American Heart Association, National Center
2002-	Chairman, Program Committee, Council on Cardiopulmonary and Critical Care, American Heart Association, National Center
2002-	Member, Committee on Scientific Sessions Program, American Heart Association
2005	Member, Respiratory Integrative Biology and Translational Research Study Section, National Heart Lung and Blood Institute
2005-	Member, Research Committee, American Heart Association
2005-	Member, Future of Scientific Sessions Task Force, American Heart Association

Professional Societies:

1997-	American Heart Association, Council on Basic Cardiovascular Science
2000	American Heart Association, Council on Cardiopulmonary and Critical Care
2001	American Society for Clinical Investigation

Editorial Boards:

1989- Ad hoc reviewer:
American Journal of Pathology
American Journal of Physiology
American Journal of Respiratory Cell and Molecular Biology
Anesthesiology
Circulation
Circulation Research
Journal of Applied Physiology
Journal of Biological Chemistry
Journal of Clinical Investigation
New England Journal of Medicine
Nature Medicine

Proceedings of the National Academy of Sciences

Awards and Honors:

1978	Magna cum laude, Brown University
1981	Patricia McCormick Memorial Prize, Brown University
1984-1986	Research Fellowship, Leukemia Society of America
1986-1987	Postdoctoral Fellowship, Pfizer Pharmaceuticals
1996-1998	Grant-in-Aid, American Heart Association, National Center (declined)
1996	Finalist, Circulation Council Cardiovascular Research Competition
2000	Second Prize for the basic science research abstracts from the Massachusetts Thoracic Society
2001	Charter Fellow of the Council on Basic Cardiovascular Sciences, American Heart Association
2002	Inaugural Fellow of the Basic Sciences Council, American Heart Association
2003	Inaugural Fellow of the Council on Cardiopulmonary, Perioperative, and Critical Care, American Heart Association

Part II: Research, Teaching, and Clinical Contributions

A. Narrative Report

Dr. Bloch's research has focused on cardiovascular biology and the molecular mechanisms regulating vascular tone and ventricular remodeling. Dr. Bloch established his own laboratory at MGH in 1991 and became a principal investigator in the Cardiovascular Research Center in 1992.

When he established his laboratory, it appeared that there was a single constitutively-expressed nitric oxide (NO) synthase (NOS) in brain and endothelium. Dr. Bloch's group cloned the NOS isoform responsible for endothelial NO production, NOS3. Dr. Bloch's group discovered that NOS3-deficient mice are more susceptible to hypoxia-induced pulmonary vascular remodeling. Enhanced pulmonary NO (either by inhalation of NO gas or by aerosol delivery of an adenovirus specifying NOS attenuates pulmonary vasoconstriction and prevents this pulmonary vascular remodeling. Moreover, they found that NO inhalation could prevent pulmonary vascular remodeling even before the development of pulmonary vasoconstriction. Taken together, these studies have important implications for the treatment of children with congenital heart disease, in whom pulmonary vascular remodeling precedes the development of pulmonary hypertension (which is often fatal). Dr. Bloch and his colleagues were the first to show that NO inhalation also has systemic vascular effects including attenuation vascular neointima formation after balloon injury and prevention re-thrombosis after coronary artery thrombolysis (the latter is markedly potentiated by inhibitors of cGMP-metabolizing phosphodiesterases). These innovative studies have direct clinical implications for the care of patients with acute coronary syndromes.

Dr. Bloch has brought into focus the fact that regulation of NO responsiveness may be as important as regulation of NO production. Dr. Bloch found that prolonged exposure of vascular smooth muscle cells (VSMC) to NO or pro-inflammatory cytokines decreases function of soluble guanylate cyclase (sGC; the enzyme responsible for cGMP synthesis in response to NO), desensitizing the cells to NO. Dr. Bloch also showed that cGMP-dependent protein kinase, an enzyme responsible for vasodilation, also has a critical role in determining the sensitivity of these cells to the antiproliferative and proapoptotic effects of NO and cGMP.

Dr. Bloch's group has contributed importantly to a second research area--the structure and function of the "nuclear body", a nuclear structure that appears to have a critical role in oncogenesis, gene transcription, and the cellular response to viral infection. They have cloned two new nuclear body components, both of which appear to be transcription factors and one of which is a co-activator of nuclear hormone receptors.

Dr. Bloch's research group has elucidated an important role for NO synthesized by endothelial nitric oxide synthase (NOS3) in the left ventricular remodeling induced by a variety of hemodynamic challenges. In addition, they have explored the mechanisms responsible for the impairment of hypoxic pulmonary vasoconstriction associated with pulmonary injury associated with endotoxemia and volutrauma.

Most recently, Dr. Bloch's group has begun a new line of research directed at understanding how mutations in the gene encoding bone morphogenetic protein receptor type 2 (BMPR2) cause primary pulmonary hypertension. They have observed that mice carrying one copy of a mutant BMPR2 gene have mild pulmonary hypertension associated with abnormalities of pulmonary vascular structure.

Dr. Bloch has also made important contributions in the clinical and research training of cardiology fellows. He is a primary research mentor for the MGH cardiology fellows guiding them to, and supporting them in, the best research training opportunities HMS has to offer. In the past five years, Dr. Bloch has played a pivotal role in the integration of the cardiology fellowship programs at the Brigham and Women's Hospital and MGH providing fellows with exposure to outstanding clinical experiences at both institutions. Since 2002, Dr. Bloch has served as principal investigator of the T32 training grant awarded to the Cardiovascular Research Center. In this role, Dr. Bloch supervises the mentoring and career development of 10 post-doctoral cardiovascular scientists each year. From 2002 through 2004, Dr. Bloch served as the Interim Director of the CVRC fostering the creativity and productivity of ten faculty members and more than 40 post-doctoral research fellows.

B. Funding Information (Research):

Past:

1991-1992	Research Grant, Dr. Louis Skarow Memorial Fund (PI: K. Bloch) "Gene expression in a model of pulmonary hypertension."
1991-1994	Grant-in-Aid, American Heart Association, National Center (PI: K. Bloch) "Pulmonary expression of endothelin genes."

1991-1996	NHLBI/R29 (PI: K. Bloch) "Biosynthesis of the endothelin family of vasoactive peptides."
1995-1996	Sponsored Research Agreement through the Cardiovascular Research Center from Bristol-Myers Squibb (PI: K. Bloch) "Isolation and characterization of novel vascular nitric oxide synthases."
1996-1997	NHLBI/R01 (Co-PI: K. Bloch) "The pulmonary response to inhaled particulates."
1996-2000	NHLBI/R01 (PI: K. Bloch) "Nitric oxide/cGMP signal transduction in pulmonary injury."
1996-2001	Established Investigator, American Heart Association, National Center (PI: K. Bloch) "Regulation of a nitric oxide receptor component, the β1 subunit of soluble guanylate cyclase."
1998-2002	NHLBI/T32 (Co-PI: K. Bloch; PI: M.C. Fishman) "Cell and molecular training for cardiovascular biology."
1998-2003	NHLBI/R01 (PI: K. Bloch) "Nitric oxide/cGMP signal transduction in vascular injury."
2001-2002	Pfizer Pharmaceuticals (Co-PI: K. Bloch; PI: M.J. Semigran) "Evaluation of the effects of sildenafil with and without inhaled nitric oxide (NO) on platelet-mediated thrombosis and cardiac function in a canine model of cyclic coronary artery occlusion."

Current:

1996-	NHLBI/R01 (Co-PI: K. Bloch; PI: W. Zapol) "Studies of inhaled nitric oxide."
2002-	INO Therapeutics, Inc. (PI: K Bloch) "Laboratory-based initiatives for the further development of the therapeutic potential of inhaled nitric oxide."
2002-	NHLBI/T32 (PI: K. Bloch) "Cell and molecular training for cardiovascular biology."
2003-	NHLBI/R01 (PI: K. Bloch) "Nitric oxide synthase 3 and left ventricular remodeling"
2003-	NHLBI/R01 (PI: K. Bloch) "BMPR2 in the pathogenesis of pulmonary hypertension"

C. Report of Current Research Activities (Bench and Clinical Research):

Project 1: Studies of inhaled nitric oxide. (Co-PI: K. Bloch)
Project 2: Evaluation of the systemic effects of breathing nitric oxide (PI: K. Bloch)
Project 3: Role of nitric oxide in left ventricular remodeling (PI: K. Bloch)
Project 4: Role of BMPR2 in the pathogenesis of pulmonary hypertension (PI: K. Bloch)
Project 5: Evaluation of nitric oxide inhalation to treat cardiogenic shock complicating right ventricular infarction. (Co-PI: K. Bloch)

D. Report of Teaching

1. Local Contributions

a. medical school

1977 — Brown University, Biomed 110, Biophysics
course director: Babette Stewart
Teaching Assistant
50 undergraduates (approx.)
3-5 hours preparation and contact time/week (approx.)
semester course

1978-1981 — Brown University, Biomed 6, Introduction to Physiology
course director: Peter Stewart
Teaching Assistant
50 undergraduates (approx.)
3-5 hours preparation and contact time/week (approx.)
semester course

1986 — Harvard University, Genetics 700.0, Fundmentals of Genetics
course director: Philip Leder
Teaching Assistant
15 medical students (approx.)
3 hours preparation and contact time/week (approx.)
semester course

1990-1992 — Harvard Medical School, Introduction to Clinical Medicine
Clinical Mentor
2-3 medical students, 3 sessions/week, 2 hours/session, 3 weeks/year
total: 9 hours preparation time, 18 hours contact time

1993-1996 — Harvard Medical School, Patient-Doctor II Course
course directors: Katherine Treadway and Diane Fingold
Preceptor
50 medical students (approx.)
3 sessions, 2 hours/session, 1/year
total: 9 hours preparation and contact time

b. graduate medical course: none

c. local invited teaching presentations

1993-1994 — "Ethical Conduct of Research" Course, Massachusetts General Hospital, Preceptor, 5-10 postdoctoral fellows, 2 sessions/year, 2 hours/session, prep time: 1 hour/session.

1994 — Cardiology Grand Rounds, Massachusetts General Hospital, Lecturer;

50 attendees: medical students, residents, clinical and research fellows, faculty; 4 hrs prep and 1 hr contact time.

1996 Anesthesiology Grand Rounds, Massachusetts General Hospital; Lecturer; 50 attendees: medical students, residents, clinical and research fellows, faculty; 4 hrs prep and 1 hr contact time.

1995 Wellman Laboratories of Photomedicine Symposium, Massachusetts General Hospital, Session Chair; 50 attendees: medical students, residents, clinical and research fellows, faculty; 1 hr prep and contact time.

1997 Cardiology Grand Rounds, Massachusetts General Hospital, Lecturer; 50 attendees: medical students, residents, clinical and research fellows, faculty; 4 hrs prep and 1 hr contact time.

1998 Clinical Fellows' Core Curriculum Lecture Series, Massachusetts General Hospital, Lecturer; 5 attendees: medical students and fellows; 4 hrs prep time and 1 hr contact time.

1999 Pulmonary and Critical Care Unit Research Conference, Massachusetts General Hospital, Lecturer; 50 attendees: medical students, residents, clinical and research fellows, faculty; 4 hrs prep and 1 hr contact time.

2000 Center for the Prevention of Cardiovascular Disease, Department of Nutrition, Harvard School of Public Health, Lecturer; 50 attendees: medical students, residents, clinical and research fellows, faculty; 4 hrs prep and 1 hr contact time.

2001 West Roxbury Veterans Administration Hospital, Cardiology Grand Rounds; 30 attendees: medical students, residents, clinical and research fellows, faculty; 4 hrs prep and 2 hr contact time.

2003 Brigham and Women's Hospital, Vascular Research Seminar; 30 attendees: medical students, residents, clinical and research fellows, faculty; 4 hrs prep and 2 hr contact time.

2004 Massachusetts General Hospital, Cardiology Grand Rounds; 50 attendees: nurses, medical students, residents, clinical and research fellows, faculty; 5 hrs prep and 1 hr contact time.

2005 Massachusetts General Hospital, Critical Care Research Retreat; 50 attendees: clinical and research fellows, faculty; 10 hours prep time, 4 hours contact time, 10 minute lecture

d. continuing medical education courses: none

e. advisory and supervisory responsibilities

1989- Attending Physician, Private and Ward Medical Services, Massachusetts General Hospital (variable hours/year)

1989-1992 Principal Investigator, Cardiovascular Research Center, Massachusetts General Hospital, scientific supervisor for Research Fellows, including one Associate Professor and one Assistant Professor of Anesthesia (1,000 hours/year).

1990- Attending Physician, Cardiology Consult Service, Massachusetts General Hospital (140 hours/year).

1991- Research Advisor, Cardiology Fellowship Program, Massachusetts

	General Hospital, 25-35 Fellows per year in clinical and research training (200 hours/year).
1992-2002	Attending Physician, Coronary Care Unit, Massachusetts General Hospital (variable hours/year).
1992-	Principal Investigator, Cardiovascular Research Center, Massachusetts General Hospital, scientific supervisor for Research Fellows, including two Assistant Professors of Anesthesia (1,000 hours/year).
2002-	Principal Investigator, NIH-sponsored program (T32) to train 10 post-doctoral cardiovascular scientists each year (200 hours/year)

f. teaching leadership role

1990-1999	Cardiac Unit Research Seminar Series, Massachusetts General Hospital; Organizer; A weekly series of presentation by staff and senior fellows designed to highlight research in the Cardiac Unit.
1995-1996	Cardiac Unit Society of Fellows, Massachusetts General Hospital; Organizer; A quarterly series of symposia presented by research fellows at the home of the Chief of the MGH Cardiac Unit.
1997	Society of Cardiology Fellows, Massachusetts General Hospital and Brigham and Women's Hospital; Co-Organizer; A quarterly series of symposia designed to foster scientific communication and collaboration between MGH and BWH and to highlight research opportunities for fellows in cardiology training.
2002-	CVRC Seminar Series; Organizer; A weekly seminar series presented by visiting scientists in the MGH Cardiovascular Research Center

g. names of advisees and trainees/current positions

1989-1990	Charles C. Hong, MD/PhD, Clinical and Research Fellow, Cardiology Division, Massachusetts General Hospital
1990-1991	Robert Schott, MD, MPH, private practice
1991-1993	Akito Shimouchi, MD, Assistant Professor of Medicine, National Cardiovascular Center Research Institute, Osaka, Japan
1990-1991	Stefan P. Janssens, MD, PhD, Associate Professor of Medicine, Cardiac Unit and Laboratory for Molecular and Vascular Biology, University Hospital Gasthuisberg, Leuven, Belgium
1992-1994, 2001-	Noriko Kawai, MD, PhD, Research Fellow in Medicine, Cardiovascular Research Center, Massachusetts General Hospital
1992-1993	John J. Lepore, MD, Instructor of Medicine, University of Pennsylvania Medical Center, Department of Medicine, Cardiovascular Medicine Division and Molecular Cardiology Laboratory
1993-1994	Johanna Wolfram, MD, University Clinic for Internal Medicine II, Department of Cardiology, General Hospital, Vienna, Austria
1993-1999	Lucienne Sanchez, MD, Instructor in Pediatrics, Harvard Medical School/Massachusetts General Hospital

1993-2004	Jesse D. Roberts, MD, Associate Professor of Anesthesiology in Pediatrics, Harvard Medical School/Massachusetts General Hospital
1994-1995	Jeffrey Thomas, MD, Associate Professor of Neurosurgery, Chief of Neurovascular and Neurointerventional Surgery, Division of Neurosurgery, The University of New Mexico Health Sciences Center
1994-1996	Alexandra Holzmann, MD, Department of Anesthesiology, University of Heidelberg
1995-2004	Heling Liu, MD, on leave to care for her children
1995-1998	Jean-Daniel Chiche, MD, Professor, Cochin University, Paris, France
1996-1997	Anita Honkanen, MD, private practice
1996-1998	Masao Takata, MD/PhD, Assistant Professor, Department of Anaesthetics and Intensive Care, Imperial College School of Medicine, Hammersmith Hospital, London, United Kingdom
1996-1998	Douglas Wirthlin, MD, Assistant Professor of Surgery, Vascular Surgery, University of Alabama at Birmingham
1996-1998	Joerg Weimann, MD, Professor of Anesthesia, Department of Anaesthesiology and Intensive Care Medicine, Charité -Berlin Medical School, Campus Benjamin Franklin
1998-2002	Galina Filippov, MD, Research Scientist, Omnigene Bioproducts Inc.
1998, 2000-2001	Zena Quezado, MD, Chief, Department of Anesthesia and Surgical Services, Warren G. Magnuson Clinical Center, National Institutes of Health
1998-2000	Roman Ullrich, MD, Associate Professor of Anesthesia and Intensive Care Medicine,Vienna General Hospital, Medical University of Vienna
1999-2001	Hiroshi Nakajima, MD, Neurosurgery Residency, Tokyo Women's Medical College, Tokyo, Japan
1999-2001	Pini Orbach, PhD, Project Manager, Drug Development, Perdix Pharmaceuticals, Inc.
1999-	Fumito Ichinose, MD, PhD, Assistant Professor of Anesthesia, Harvard Medical School/Massachusetts General Hospital
2001-2003	Aimee Limbach, PhD, Post-doctoral Fellow, Center for Human Molecular Genetics, Munroe-Meyer Institute, University of Nebraska Medical Center
2002-2003	Elisabeth Choe, MD, Resident in Internal Medicine, University of Texas Southwestern
2002-2004	Cornelius Busch, MD, Resident in Anesthesia, Department of Anesthesiology, University of Heidelberg
2003	Claire Mayeur, MD, Resident in Anesthesiology, Lille, France
2003-	Hideyuki Beppu, MD, PhD, Instructor in Medicine, Cardiovascular Research Center, Massachusetts General Hospital
2003-	Manu Buys, PhD, Research Fellow in Medicine, Cardiovascular Research Center, Massachusetts General Hospital

2003-	Paul Yu, MD, PhD, Clinical and Research Fellow in Medicine, Cardiovascular Research Center, Massachusetts General Hospital
2004-2005	David Bayne, undergraduate student, Harvard University
2004-	Ryuji Hataishi, MD, PhD, Research Fellow in Anesthesia, Massachusetts General Hospital
2004-	Rajeev Malhotra, MD, Resident in Internal Medicine, Massachusetts General Hospital
2004-	Tomas Neilan, MD, Clinical and Research Fellow in Medicine, Cardiac Ultrasound Laboratory and Cardiovascular Research Center, Massachusetts General Hospital
2005-	Sarah Blake, MD, Research Fellow in Anesthesia, Massachusetts General Hospital

2. Regional, National, or International Contributions

1994	Invited Lecturer, American College of Cardiology, Dallas, TX
1994	Invited Lecturer, Pfizer Pharmaceuticals, Groton, CT
1994	Invited Lecturer, University of Leuven, Belgium
1995	Invited Lecturer, St. Elizabeth's Hospital, Cardiovascular Research Seminar, Boston, MA
1996	Invited Lecturer, Boston Heart Foundation, Boston, MA
1996	Invited Lecturer, Georgia Medical College, Vascular Biology Division, Atlanta, GA
1997	Invited Lecturer, Harvard Medical School, Vascular Biology Seminar, Boston, MA
1997	Invited Lecturer, Boston University, Whittaker Foundation, Boston, MA
1998	Invited Lecturer, Oregon Health Sciences University, Cardiology Division, Oregon
1998	Invited Lecturer, Brigham and Women's Hospital, Cardiology Division, Monday Morning Research Conference, Boston, MA
1999	Invited Lecturer, New York Medical College, Department of Pharmacology Seminar, New York, NY
1999	Invited Lecturer, Tufts University School of Medicine, Dept. of Medicine, Boston, MA
1999	Invited Lecturer, Tufts University School of Medicine/New England Medical Center, Pulmonary and Critical Care Division, Boston, MA
1999	Invited Lecturer, Millenium Pharmaceuticals, Inc., Cambridge, MA
1999	Invited Lecturer, University of Washington, Cardiology Dept., Seattle, WA
1999	Invited Lecturer, University of Alabama at Birmingham, Dept. of Pathology, Birmingham, AL
1999	Invited Lecturer, 3rd International Society for Medical Gases Meeting, Heidelberg, Germany
2004	Invited Lecturer, National Institute of Health, Critical Care Medicine, Bethesda, MD
	Invited Lecturer, INO Therapeutics Inc. Scientific Advisory Board, Chatham, MA

2002	Invited Lecturer, Medical College of Wisconsin, Milwaukee, Wisconsin
2002	Invited Lecturer, American Heart Association Scientific Sessions, Chicago, IL
2003	Invited Lecturer, Department for Molecular and Biomedical Research, Universiteit Gent, Belgium
2003	Invited Lecturer, Whitaker Cardiovascular Institute, Boston University Medical School
2003	Invited Lecturer, Cardiology Division, University of Alberta, Edmonton, Canada
2004	Invited Lecturer, American Heart Association, Northeast Affiliate, Symposium: Launching a Career in Cardiovascular Research
2004	Invited Lecturer, Cardiology Grand Rounds, Dartmouth-Hitchcock Medical Center—"Nitric oxide synthases in ventricular remodeling: insights gained from genetically-modified mice."
2004	Invited Lecturer, Vascular Biology Seminar, Dartmouth-Hitchcock Medical Center—"Mechanisms regulating pulmonary vascular structure and function—roles of leukotrienes and bone morphogenetic proteins."
2004	Invited Lecturer, Department of Physiology Seminar, Louisiana State University-Shreveport—"Nitric oxide synthases in ventricular remodeling: insights gained from genetically-modified mice."
2004	Invited Lecturer, Cardiovascular Cell and Gene Therapy Conference II, Cambridge, MA—"Nitric oxide/cGMP signal transduction—implications for cardiovascular gene transfer."
2004	Invited Lecturer, Critical Therapeutics, Inc., Lexington, MA—"Mechanisms of pulmonary vascular dysfunction in lung injury: insights gained from genetically-modified mice."

E. Report of Clinical Activities

Dr. Bloch is a practicing cardiologist who maintains a practice within the Cardiac Unit Associates and Cardiology Division at the Massachusetts General Hospital. His practice consists of patients with cardiac problems of a moderate to high level of complexity referred to a tertiary care center.

Part III: Bibliography

Original Articles:

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3. Graham RM, Bloch KD, Delaney VB, Bourke E, Seidman JG. Bartter's syndrome and the atrial natriuretic factor gene. Hypertension 1986, 8:549-551.

4. Ballerman BJ, Bloch KD, Seidman JG, Brenner BM. Atrial natriuretic peptide transcription, secretion, and glomerular receptor activity during mineralocorticoid escape. J Clin Invest 1986, 78:840-843.

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6,601,580: Enhancing therapeutic effectiveness of nitric oxide inhalation.
6,656,452: Use of inhaled NO as anti-inflammatory agent.
6,720,309: Method of inducing vasodilation and treating pulmonary hypertension using adenoviral-mediated transfer of the nitric oxide synthase gene.
6,811,768: Use of inhaled NO as anti-inflammatory agent.

EXHIBIT B

EXHIBIT B

FIGURE 1

Alignment of human DNMT3A from ATCC Deposit No. 98809 (top) and currently amended SEQ ID NO:3 (bottom)[1]

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gccgcggcaccagggcgcgcagccgggccggcccgaccccaccggccatacggtggagcc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gccgcggcaccagggcgcgcagccgggccggcccgaccccaccggccatacggtggagcc 60

atcgaagcccccacccacaggctgacagaggcaccgttcaccagagggctcaacaccggg
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
atcgaagcccccacccacaggctgacagaggcaccgttcaccagagggctcaacaccggg 120

atctatgtttaagttttaactctcgcctccaaagaccacgataattccttccccaaagcc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
atctatgtttaagttttaactctcgcctccaaagaccacgataattccttccccaaagcc 180

cagcagccccccagccccgcgcagccccagcctgcctcccggcgcccagatgcccgccat
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cagcagccccccagccccgcgcagccccagcctgcctcccggcgcccagatgcccgccat 240

gccctccagcggccccggggacaccagcagctctgctgcggagcgggaggaggaccgaaa
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gccctccagcggccccggggacaccagcagctctgctgcggagcgggaggaggaccgaaa 300

ggacggagaggagcaggaggagccgcgtggcaaggaggagcgccaagagcccagcaccac
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ggacggagaggagcaggaggagccgcgtggcaaggaggagcgccaagagcccagcaccac 360

ggcacggaaggtggggcggcctgggaggaagcgcaagcacccccggtggaaagcggtga
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cacgccaaaggaccctgcggtgatctccaagtccccatccatggcccaggactcaggcgc
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ctcagagctattacccaatggggacttggagaagcggagtgagccccagccagaggag..
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ctcagagctattacccaatggggacttggagaagcggagtgagccccagccagaggaggg 540

..............................................agggtgcagctgagac
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gagccctgctgggggcagaagggcggggccccagcagagggagagggtgcagctgagac 600

cctgcctgaagcctcaagagcagtggaaaatggctgctgcacccccaaggagggccgagg
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cctgcctgaagcctcaagagcagtggaaaatggctgctgcacccccaaggagggccgagg 660

agcccctgcagaagcgggcaaagaacagaaggagaccaacatcgaatccatgaaaatgga
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
agcccctgcagaagcgggcaaagaacagaaggagaccaacatcgaatccatgaaaatgga 720
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[1] Bolded nucleotides indicate nucleotides that were amended on July 23, 2001.

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gggctcccggggccggctgcggggtggcttgggctgggagtccagcctccgtcagcggcc
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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catgccgaggctcaccttccaggcgggggacccctactacatcagcaagcgcaagcggga
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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cgagtggctggcacgctggaaaagggaggctgagaagaaagccaaggtcattgcaggaat
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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gaatgctgtggaagaaaaccaggggcccggggagtctcagaaggtggaggaggccagccc
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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gctgaaggagtattttgcgtgtgtgtaagggacatgggggcaaactgaggtagcg
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gctgaaggagtattttgcgtgtgtgtaagggacatgggggcaaactgaggtagcg 2995
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FIGURE 2

Alignment of predicted amino acids encoded by DNMT3A cDNA in ATCC Deposit No. 98809 (top) and predicted amino acids encoded by currently amended SEQ ID NO:3 (bottom)[2]

MPAMPSSGPGDTSSSAAEREEDRKDGEEQEEPRGKEERQEPSTTARKVGRPGRKR	55
MPAMPSSGPGDTSSSAAEREEDRKDGEEQEEPRGKEERQEPSTTARKVGRPGRKR	55
KHPPVESGDTPKDPAVISKSPSMAQDSGASELLPNGDLEKRSEPQPEE**RVQLRPC**	110
KHPPVESGDTPKDPAVISKSPSMAQDSGASELLPNGDLEKRSEPQPEEGSPAGGQ	110
LKPQEQWKMAAAPPRRAEEPLQKRAKNRRRPTSNP*KWRAPGAGCGVAWAGSPAS	165
KGGAPAEGEGAAETLPEASRAVENGCCTPKEGRGAPAEAGKEQKETNIESMKMEG	165
VSGPCRGSPSRRGTPTTSASASGTSGWHAGKGRLRRKPRSLQE*MLWKKTRGPGS	220
SRGRLRGGLGWESSLRQRPMPRLTFQAGDPYYISKRKRDEWLARWKREAEKKAKV	220
LRRWRRPALLLCSSPLTPHPPLWLPRLSPWGPMLGTRMPPKQAMTSQSTRTAGAL	275
GMNAVEENQGPGESQKVEEASPPAVQQPTDPASPTVATTPEPVGSDAGDKNIAAT	275
ALGSWCGGNCGASPGGQAALCLGG*RAGAEQLKAPAGSCGSETANSQWCVLRS*C	330
KAGDDEPEYEDGRGFGIGELVWGKLRGFSWWPGRIVSWWMTGRSRAAEGTRWVMW	330
R*ARFAVRSTRPRTTSSPCTAKPSTRSCRWPAAARGSCSRCATTAMRVTLPRPWR	385
FGDGKFSVVCVEKLMPLSSFCSAFHQATYNKQPMYRKAIYEVLQVASSRAGKLFP	385
CRTSP*LNGPWGASSLLALRAWSHQKKRRIPTKKCTRTCGWNLRQLPTHHLHQPK	440
VCHDSDESDTAKAVEVQNKPMIEWALGGFQPSGPKGLEPPEEEKNPYKEVYTDMW	440
SPGRAQRRSPRSRRLLMSAQESGWCTRCGRSAGTLRTSASPVGASMLPWNTPSSL	495
VEPEAAAYAPPPPAKKPRKSTAEKPKVKEIIDERTRERLVYEVRQKCRNIEDICI	495
EECAKTARTAFWSVRTSTTTTATSPTAPSAVGAVRCSCAETTTAAGAFAWSVWTS	550
SCGSLNVTLEHPLFVGGMCQNCKNCFLECAYQYDDDGYQSYCTICCGGREVLMCG	550
WWGRGLPRQPLRKTPGTATCAGTRVPTGCCGGERTGPPGSRCSSLITTTRNLTLQ	605
NNNCCRCFCVECVDLLVGPGAAQAAIKEDPWNCYMCGHKGTYGLLRRREDWPSRL	605
RFTHLSQLRRGSPSGCCLSLMESLQGSWC*RTWAFRWTATLPRRCVRTPSRWAWC	660
QMFFANNHDQEFDPPKVYPPVPAEKRKPIRVLSLFDGIATGLLVLKDLGIQVDRY	660
GTRGRSCTSGTSAASHRSISRSGAHSIW*LGAVPAMTSPSSTLLARASTRALAGS	715
IASEVCEDSITVGMVRHQGKIMYVGDVRSVTQKHIQEWGPFDLVIGGSPCNDLSI	715
SLSSTASCMMRGPRREMIAPSSGSLRMWWPWALVTRGTSRDFSSPTLLMPKKC**	770
VNPARKGLYEGTGRLFFEFYRLLHDARPKEGDDRPFFWLFENVVAMGVSDKRDIS	770
QLHTGPATSGVTFPV*TGRWHPL*MISWSCRSVWSMAG*PSSAK*GPLLRGQTP*	825
RFLESNPVMIDAKEVSAAHRARYFWGNLPGMNRPLASTVNDKLELQECLEHGRIA	825

[2] * indicates a predicted stop codon. Bolded amino acids are encoded by nucleotides located downstream of the deletion.

SRAKTSIFLSS*MRKRTSYGALKWKGYLVSQSTILTSPT*AAWRGRDCWAGHGAC 880
KFSKVRTITTRSNSIKQGKDQHFPVFMNEKEDILWCTEMERVFGFPVHYTDVSNM 880

QSSATSSLR*RSILRVCKGHGGKLRAAWRG** 912
SRLARQRLLGRSWSVPVIRHLFAPLKEYFACV 912

FIGURE 3

Human DNMT3A from ATCC Deposit No. 98809 with forward reading frames (DNMT3A amino acid residues bolded)

```
DNA: GCCGCGGCACCAGGGCGCGCAGCCGGGCCGGCCCGACCCCACCGGCCATAC      51
+3:    R  G  T  R  A  R  S  R  A  G  P  T  P  P  A  I  R
+2:   P  R  H  Q  G  A  Q  P  G  R  P  D  P  T  G  H  T
+1:  A  A  A  P  G  R  A  A  G  P  A  R  P  H  R  P  Y

DNA: GGTGGAGCCATCGAAGCCCCCACCCACAGGCTGACAGAGGCACCGTTCACC     102
+3:    W  S  H  R  S  P  H  P  Q  A  D  R  G  T  V  H  Q
+2:   V  E  P  S  K  P  P  P  T  G  *  Q  R  H  R  S  P
+1:  G  G  A  I  E  A  P  T  H  R  L  T  E  A  P  F  T

DNA: AGAGGGCTCAACACCGGGATCTATGTTTAAGTTTTAACTCTCGCCTCCAAA     153
+3:    R  A  Q  H  R  D  L  C  L  S  F  N  S  R  L  Q  R
+2:   E  G  S  T  P  G  S  M  F  K  F  *  L  S  P  P  K
+1:  R  G  L  N  T  G  I  Y  V  *  V  L  T  L  A  S  K

DNA: GACCACGATAATTCCTTCCCCAAAGCCCAGCAGCCCCCCAGCCCCGCGCAG     204
+3:    P  R  *  F  L  P  Q  S  P  A  A  P  Q  P  R  A  A
+2:   T  T  I  I  P  S  P  K  P  S  S  P  P  A  P  R  S
+1:  D  H  D  N  S  F  P  K  A  Q  Q  P  P  S  P  A  Q

DNA: CCCCAGCCTGCCTCCCGGCGCCCAGATGCCCGCCATGCCCTCCAGCGGCCC     255
+3:    P  A  C  L  P  A  P  R  C  P  P  C  P  P  A  A  P
+2:   P  S  L  P  P  G  A  Q  M  P  A  M  P  S  S  G  P
+1:  P  Q  P  A  S  R  R  P  D  A  R  H  A  L  Q  R  P

DNA: CGGGGACACCAGCAGCTCTGCTGCGGAGCGGGAGGAGGACCGAAAGGACGG     306
+3:    G  T  P  A  A  L  L  R  S  G  R  R  T  E  R  T  E
+2:   G  D  T  S  S  S  A  A  E  R  E  E  D  R  K  D  G
+1:  R  G  H  Q  Q  L  C  C  G  A  G  G  G  P  K  G  R

DNA: AGAGGAGCAGGAGGAGCCGCGTGGCAAGGAGGAGCGCCAAGAGCCCAGCAC     357
+3:    R  S  R  R  S  R  V  A  R  R  S  A  K  S  P  A  P
+2:   E  E  Q  E  E  P  R  G  K  E  E  R  Q  E  P  S  T
+1:  R  G  A  G  G  A  A  W  Q  G  G  A  P  R  A  Q  H

DNA: CACGGCACGGAAGGTGGGGCGGCCTGGGAGGAAGCGCAAGCACCCCCCGGT     408
+3:    R  H  G  R  W  G  G  L  G  G  S  A  S  T  P  R  W
+2:   T  A  R  K  V  G  R  P  G  R  K  R  K  H  P  P  V
+1:  H  G  T  E  G  G  A  A  W  E  E  A  Q  A  P  P  G

DNA: GGAAAGCGGTGACACGCCAAAGGACCCTGCGGTGATCTCCAAGTCCCCATC     459
+3:    K  A  V  T  R  Q  R  T  L  R  *  S  P  S  P  H  P
+2:   E  S  G  D  T  P  K  D  P  A  V  I  S  K  S  P  S
+1:  G  K  R  *  H  A  K  G  P  C  G  D  L  Q  V  P  I

DNA: CATGGCCCAGGACTCAGGCGCCTCAGAGCTATTACCCAATGGGGACTTGGA     510
+3:    W  P  R  T  Q  A  P  Q  S  Y  Y  P  M  G  T  W  R
+2:   M  A  Q  D  S  G  A  S  E  L  L  P  N  G  D  L  E
+1:  H  G  P  G  L  R  R  L  R  A  I  T  Q  W  G  L  G

DNA: GAAGCGGAGTGAGCCCCAGCCAGAGGAG.....                      561
+3:    S  G  V  S  P  S  Q  R  R.....
+2:   K  R  S  E  P  Q  P  E  E.....DELETION.....
+1:  E  A  E  *  A  P  A  R  G  E.....
```

```
DNA: .....AGGGTGCAGCTGAGACCCTGCCTGAAGC                    612
+3:  .....G  C  S  *  D  P  A  *  S
+2:  .....R  V  Q  L  R  P  C  L  K  P
+1:  .....G  A  A  E  T  L  P  E  A

DNA: CTCAAGAGCAGTGGAAAATGGCTGCTGCACCCCCAAGGAGGGCCGAGGAGC  663
+3:  L  K  S  S  G  K  W  L  L  H  P  Q  G  G  P  R  S
+2:  Q  E  Q  W  K  M  A  A  A  P  P  R  R  A  E  E  P
+1:  S  R  A  V  E  N  G  C  C  T  P  K  E  G  R  G  A

DNA: CCCTGCAGAAGCGGGCAAAGAACAGAAGGAGACCAACATCGAATCCATGAA  714
+3:  P  C  R  S  G  Q  R  T  E  G  D  Q  H  R  I  H  E
+2:  L  Q  K  R  A  K  N  R  R  R  P  T  S  N  P  *  K
+1:  P  A  E  A  G  K  E  Q  K  E  T  N  I  E  S  M  K

DNA: AATGGAGGGCTCCCGGGGCCGGCTGCGGGGTGGCTTGGGCTGGGAGTCCAG  765
+3:  N  G  G  L  P  G  P  A  A  G  W  L  G  L  G  V  Q
+2:  W  R  A  P  G  A  G  C  G  V  A  W  A  G  S  P  A
+1:  M  E  G  S  R  G  R  L  R  G  G  L  G  W  E  S  S

DNA: CCTCCGTCAGCGGCCCATGCCGAGGCTCACCTTCCAGGCGGGGGACCCCTA  816
+3:  P  P  S  A  A  H  A  E  A  H  L  P  G  G  G  P  L
+2:  S  V  S  G  P  C  R  G  S  P  S  R  R  G  T  P  T
+1:  L  R  Q  R  P  M  P  R  L  T  F  Q  A  G  D  P  Y

DNA: CTACATCAGCAAGCGCAAGCGGGACGAGTGGCTGGCACGCTGGAAAAGGGA  867
+3:  L  H  Q  Q  A  Q  A  G  R  V  A  G  T  L  E  K  G
+2:  T  S  A  S  A  S  G  T  S  G  W  H  A  G  K  G  R
+1:  Y  I  S  K  R  K  R  D  E  W  L  A  R  W  K  R  E

DNA: GGCTGAGAAGAAAGCCAAGGTCATTGCAGGAATGAATGCTGTGGAAGAAAA  918
+3:  G  *  E  E  S  Q  G  H  C  R  N  E  C  C  G  R  K
+2:  L  R  R  K  P  R  S  L  Q  E  *  M  L  W  K  K  T
+1:  A  E  K  K  A  K  V  I  A  G  M  N  A  V  E  E  N

DNA: CCAGGGGCCCGGGGAGTCTCAGAAGGTGGAGGAGGCCAGCCCTCCTGCTGT  969
+3:  P  G  A  R  G  V  S  E  G  G  G  G  Q  P  S  C  C
+2:  R  G  P  G  S  L  R  R  W  R  R  P  A  L  L  L  C
+1:  Q  G  P  G  E  S  Q  K  V  E  E  A  S  P  P  A  V

DNA: GCAGCAGCCCACTGACCCCGCATCCCCCACTGTGGCTACCACGCCTGAGCC  1020
+3:  A  A  A  H  *  P  R  I  P  H  C  G  Y  H  A  *  A
+2:  S  S  P  L  T  P  H  P  P  L  W  L  P  R  L  S  P
+1:  Q  Q  P  T  D  P  A  S  P  T  V  A  T  T  P  E  P

DNA: CGTGGGGTCCGATGCTGGGGACAAGAATGCCACCAAAGCAGGCGATGACGA  1071
+3:  R  G  V  R  C  W  G  Q  E  C  H  Q  S  R  R  *  R
+2:  W  G  P  M  L  G  T  R  M  P  P  K  Q  A  M  T  S
+1:  V  G  S  D  A  G  D  K  N  A  T  K  A  G  D  D  E

DNA: GCCAGAGTACGAGGACGGCCGGGGCTTTGGCATTGGGGAGCTGGTGTGGGG  1122
+3:  A  R  V  R  G  R  P  G  L  W  H  W  G  A  G  V  G
+2:  Q  S  T  R  T  A  G  A  L  A  L  G  S  W  C  G  G
+1:  P  E  Y  E  D  G  R  G  F  G  I  G  E  L  V  W  G

DNA: GAAACTGCGGGGCTTCTCCTGGTGGCCAGGCCGCATTGTGTCTTGGTGGAT  1173
+3:  E  T  A  G  L  L  L  V  A  R  P  H  C  V  L  V  D
+2:  N  C  G  A  S  P  G  G  Q  A  A  L  C  L  G  G  *
+1:  K  L  R  G  F  S  W  W  P  G  R  I  V  S  W  W  M
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```
DNA: GACGGGCCGGAGCCGAGCAGCTGAAGGCACCCGCTGGGTCATGTGGTTCGG    1224
+3:  D  G  P  E  P  S  S  *  R  H  P  L  G  H  V  V  R
+2:     R  A  G  A  E  Q  L  K  A  P  A  G  S  C  G  S  E
+1:     T  G  R  S  R  A  A  E  G  T  R  W  V  M  W  F  G

DNA: AGACGGCAAATTCTCAGTGGTGTGTGTTGAGAAGCTGATGCCGCTGAGCTC    1275
+3:  R  R  Q  I  L  S  G  V  C  *  E  A  D  A  A  E  L
+2:     T  A  N  S  Q  W  C  V  L  R  S  *  C  R  *  A  R
+1:     D  G  K  F  S  V  V  C  V  E  K  L  M  P  L  S  S

DNA: GTTTTGCAGTGCGTTCCACCAGGCCACGTACAACAAGCAGCCCATGTACCG    1326
+3:  V  L  Q  C  V  P  P  G  H  V  Q  Q  A  A  H  V  P
+2:     F  A  V  R  S  T  R  P  R  T  T  S  S  P  C  T  A
+1:     F  C  S  A  F  H  Q  A  T  Y  N  K  Q  P  M  Y  R

DNA: CAAAGCCATCTACGAGGTCCTGCAGGTGGCCAGCAGCCGCGCGGGGAAGCT    1377
+3:  Q  S  H  L  R  G  P  A  G  G  Q  Q  P  R  G  E  A
+2:     K  P  S  T  R  S  C  R  W  P  A  A  A  R  G  S  C
+1:     K  A  I  Y  E  V  L  Q  V  A  S  S  R  A  G  K  L

DNA: GTTCCCGGTGTGCCACGACAGCGATGAGAGTGACACTGCCAAGGCCGTGGA    1428
+3:  V  P  G  V  P  R  Q  R  *  E  *  H  C  Q  G  R  G
+2:     S  R  C  A  T  T  A  M  R  V  T  L  P  R  P  W  R
+1:     F  P  V  C  H  D  S  D  E  S  D  T  A  K  A  V  E

DNA: GGTGCAGAACAAGCCCATGATTGAATGGGCCCTGGGGGGCTTCCAGCCTTC    1479
+3:  G  A  E  Q  A  H  D  *  M  G  P  G  G  L  P  A  F
+2:     C  R  T  S  P  *  L  N  G  P  W  G  A  S  S  L  L
+1:     V  Q  N  K  P  M  I  E  W  A  L  G  G  F  Q  P  S

DNA: TGGCCCTAAGGGCCTGGAGCCACCAGAAGAAGAGAAGAATCCCTACAAAGA    1530
+3:  W  P  *  G  P  G  A  T  R  R  R  E  E  S  L  Q  R
+2:     A  L  R  A  W  S  H  Q  K  K  R  R  I  P  T  K  K
+1:     G  P  K  G  L  E  P  P  E  E  E  K  N  P  Y  K  E

DNA: AGTGTACACGGACATGTGGGTGGAACCTGAGGCAGCTGCCTACGCACCACC    1581
+3:  S  V  H  G  H  V  G  G  T  *  G  S  C  L  R  T  T
+2:     C  T  R  T  C  G  W  N  L  R  Q  L  P  T  H  H  L
+1:     V  Y  T  D  M  W  V  E  P  E  A  A  A  Y  A  P  P

DNA: TCCACCAGCCAAAAAGCCCCGGAAGAGCACAGCGGAGAAGCCCAAGGTCAA    1632
+3:  S  T  S  Q  K  A  P  E  E  H  S  G  E  A  Q  G  Q
+2:     H  Q  P  K  S  P  G  R  A  Q  R  R  S  P  R  S  R
+1:     P  P  A  K  K  P  R  K  S  T  A  E  K  P  K  V  K

DNA: GGAGATTATTGATGAGCGCACAAGAGAGCGGCTGGTGTACGAGGTGCGGCA    1683
+3:  G  D  Y  *  *  A  H  K  R  A  A  G  V  R  G  A  A
+2:     R  L  L  M  S  A  Q  E  S  G  W  C  T  R  C  G  R
+1:     E  I  I  D  E  R  T  R  E  R  L  V  Y  E  V  R  Q

DNA: GAAGTGCCGGAACATTGAGGACATCTGCATCTCCTGTGGGAGCCTCAATGT    1734
+3:  E  V  P  E  H  *  G  H  L  H  L  L  W  E  P  Q  C
+2:     S  A  G  T  L  R  T  S  A  S  P  V  G  A  S  M  L
+1:     K  C  R  N  I  E  D  I  C  I  S  C  G  S  L  N  V

DNA: TACCCTGGAACACCCCCTCTTCGTTGGAGGAATGTGCCAAAACTGCAAGAA    1785
+3:  Y  P  G  T  P  P  L  R  W  R  N  V  P  K  L  Q  E
+2:     P  W  N  T  P  S  S  L  E  E  C  A  K  T  A  R  T
+1:     T  L  E  H  P  L  F  V  G  G  M  C  Q  N  C  K  N
```

```
DNA: CTGCTTTCTGGAGTGTGCGTACCAGTACGACGACGACGGCTACCAGTCCTA    1836
+3:  L  L  S  G  V  C  V  P  V  R  R  R  R  L  P  V  L
+2:    A  F  W  S  V  R  T  S  T  T  T  T  A  T  S  P  T
+1:   C  F  L  E  C  A  Y  Q  Y  D  D  D  G  Y  Q  S  Y

DNA: CTGCACCATCTGCTGTGGGGGCCGTGAGGTGCTCATGTGCGGAAACAACAA    1887
+3:  L  H  H  L  L  W  G  P  *  G  A  H  V  R  K  Q  Q
+2:    A  P  S  A  V  G  A  V  R  C  S  C  A  E  T  T  T
+1:   C  T  I  C  C  G  G  R  E  V  L  M  C  G  N  N  N

DNA: CTGCTGCAGGTGCTTTTGCGTGGAGTGTGTGGACCTCTTGGTGGGGCCGGG    1938
+3:  L  L  Q  V  L  L  R  G  V  C  G  P  L  G  G  A  G
+2:    A  A  G  A  F  A  W  S  V  W  T  S  W  W  G  R  G
+1:   C  C  R  C  F  C  V  E  C  V  D  L  L  V  G  P  G

DNA: GGCTGCCCAGGCAGCCATTAAGGAAGACCCCTGGAACTGCTACATGTGCGG    1989
+3:  G  C  P  G  S  H  *  G  R  P  L  E  L  L  H  V  R
+2:    L  P  R  Q  P  L  R  K  T  P  G  T  A  T  C  A  G
+1:   A  A  Q  A  A  I  K  E  D  P  W  N  C  Y  M  C  G

DNA: GCACAAGGGTACCTACGGGCTGCTGCGGCGGCGAGAGGACTGGCCCTCCCG    2040
+3:  A  Q  G  Y  L  R  A  A  A  A  A  R  G  L  A  L  P
+2:    T  R  V  P  T  G  C  C  G  G  E  R  T  G  P  P  G
+1:   H  K  G  T  Y  G  L  L  R  R  R  E  D  W  P  S  R

DNA: GCTCCAGATGTTCTTCGCTAATAACCACGACCAGGAATTTGACCCTCCAAA    2091
+3:  A  P  D  V  L  R  *  *  P  R  P  G  I  *  P  S  K
+2:    S  R  C  S  S  L  I  T  T  T  R  N  L  T  L  Q  R
+1:   L  Q  M  F  F  A  N  N  H  D  Q  E  F  D  P  P  K

DNA: GGTTTACCCACCTGTCCCAGCTGAGAAGAGGAAGCCCATCCGGGTGCTGTC    2142
+3:  G  L  P  T  C  P  S  *  E  E  E  A  H  P  G  A  V
+2:    F  T  H  L  S  Q  L  R  R  G  S  P  S  G  C  C  L
+1:   V  Y  P  P  V  P  A  E  K  R  K  P  I  R  V  L  S

DNA: TCTCTTTGATGGAATCGCTACAGGGCTCCTGGTGCTGAAGGACTTGGGCAT    2193
+3:  S  L  *  W  N  R  Y  R  A  P  G  A  E  G  L  G  H
+2:    S  L  M  E  S  L  Q  G  S  W  C  *  R  T  W  A  F
+1:   L  F  D  G  I  A  T  G  L  L  V  L  K  D  L  G  I

DNA: TCAGGTGGACCGCTACATTGCCTCGGAGGTGTGTGAGGACTCCATCACGGT    2244
+3:  S  G  G  P  L  H  C  L  G  G  V  *  G  L  H  H  G
+2:    R  W  T  A  T  L  P  R  R  C  V  R  T  P  S  R  W
+1:   Q  V  D  R  Y  I  A  S  E  V  C  E  D  S  I  T  V

DNA: GGGCATGGTGCGGCACCAGGGGAAGATCATGTACGTCGGGGACGTCCGCAG    2295
+3:  G  H  G  A  A  P  G  E  D  H  V  R  R  G  R  P  Q
+2:    A  W  C  G  T  R  G  R  S  C  T  S  G  T  S  A  A
+1:   G  M  V  R  H  Q  G  K  I  M  Y  V  G  D  V  R  S

DNA: CGTCACACAGAAGCATATCCAGGAGTGGGGCCCATTCGATCTGGTGATTGG    2346
+3:  R  H  T  E  A  Y  P  G  V  G  P  I  R  S  G  D  W
+2:    S  H  R  S  I  S  R  S  G  A  H  S  I  W  *  L  G
+1:   V  T  Q  K  H  I  Q  E  W  G  P  F  D  L  V  I  G

DNA: GGGCAGTCCCTGCAATGACCTCTCCATCGTCAACCCTGCTCGCAAGGGCCT    2397
+3:  G  Q  S  L  Q  *  P  L  H  R  Q  P  C  S  Q  G  P
+2:    A  V  P  A  M  T  S  P  S  S  T  L  L  A  R  A  S
+1:   G  S  P  C  N  D  L  S  I  V  N  P  A  R  K  G  L
```

```
DNA: CTACGAGGGCACTGGCCGGCTCTTCTTTGAGTTCTACCGCCTCCTGCATGA    2448
+3:  L  R  G  H  W  P  A  L  L  *  V  L  P  P  P  A  *
+2:    T  R  A  L  A  G  S  S  L  S  S  T  A  S  C  M  M
+1:   Y  E  G  T  G  R  L  F  F  E  F  Y  R  L  L  H  D

DNA: TGCGCGGCCCAAGGAGGGAGATGATCGCCCCTTCTTCTGGCTCTTTGAGAA    2499
+3:  C  A  A  Q  G  G  R  *  S  P  L  L  L  A  L  *  E
+2:    R  G  P  R  R  E  M  I  A  P  S  S  G  S  L  R  M
+1:   A  R  P  K  E  G  D  D  R  P  F  F  W  L  F  E  N

DNA: TGTGGTGGCCATGGGCGTTAGTGACAAGAGGGACATCTCGCGATTTCTCGA    2550
+3:  C  G  G  H  G  R  *  *  Q  E  G  H  L  A  I  S  R
+2:    W  W  P  W  A  L  V  T  R  G  T  S  R  D  F  S  S
+1:   V  V  A  M  G  V  S  D  K  R  D  I  S  R  F  L  E

DNA: GTCCAACCCTGTGATGATTGATGCCAAAGAAGTGTCAGCTGCACACAGGGC    2601
+3:  V  Q  P  C  D  D  *  C  Q  R  S  V  S  C  T  Q  G
+2:    P  T  L  *  *  L  M  P  K  K  C  Q  L  H  T  G  P
+1:   S  N  P  V  M  I  D  A  K  E  V  S  A  A  H  R  A

DNA: CCGCTACTTCTGGGGTAACCTTCCCGGTATGAACAGGCCGTTGGCATCCAC    2652
+3:  P  L  L  L  G  *  P  S  R  Y  E  Q  A  V  G  I  H
+2:    A  T  S  G  V  T  F  P  V  *  T  G  R  W  H  P  L
+1:   R  Y  F  W  G  N  L  P  G  M  N  R  P  L  A  S  T

DNA: TGTGAATGATAAGCTGGAGCTGCAGGAGTGTCTGGAGCATGGCAGGATAGC    2703
+3:  C  E  *  *  A  G  A  A  G  V  S  G  A  W  Q  D  S
+2:    *  M  I  S  W  S  C  R  S  V  W  S  M  A  G  *  P
+1:   V  N  D  K  L  E  L  Q  E  C  L  E  H  G  R  I  A

DNA: CAAGTTCAGCAAAGTGAGGACCATTACTACGAGGTCAAACTCCATAAAGCA    2754
+3:  Q  V  Q  Q  S  E  D  H  Y  Y  E  V  K  L  H  K  A
+2:    S  S  A  K  *  G  P  L  L  R  G  Q  T  P  *  S  R
+1:   K  F  S  K  V  R  T  I  T  T  R  S  N  S  I  K  Q

DNA: GGGCAAAGACCAGCATTTTCCTGTCTTCATGAATGAGAAAGAGGACATCTT    2805
+3:  G  Q  R  P  A  F  S  C  L  H  E  *  E  R  G  H  L
+2:    A  K  T  S  I  F  L  S  S  *  M  R  K  R  T  S  Y
+1:   G  K  D  Q  H  F  P  V  F  M  N  E  K  E  D  I  L

DNA: ATGGTGCACTGAAATGGAAAGGGTATTTGGTTTCCCAGTCCACTATACTGA    2856
+3:  M  V  H  *  N  G  K  G  I  W  F  P  S  P  L  Y  *
+2:    G  A  L  K  W  K  G  Y  L  V  S  Q  S  T  I  L  T
+1:   W  C  T  E  M  E  R  V  F  G  F  P  V  H  Y  T  D

DNA: CGTCTCCAACATGAGCCGCTTGGCGAGGCAGAGACTGCTGGGCCGGTCATG    2907
+3:  R  L  Q  H  E  P  L  G  E  A  E  T  A  G  P  V  M
+2:    S  P  T  *  A  A  W  R  G  R  D  C  W  A  G  H  G
+1:   V  S  N  M  S  R  L  A  R  Q  R  L  L  G  R  S  W

DNA: GAGCGTGCCAGTCATCCGCCACCTCTTCGCTCCGCTGAAGGAGTATTTTGC    2958
+3:  E  R  A  S  H  P  P  P  L  R  S  A  E  G  V  F  C
+2:    A  C  Q  S  S  A  T  S  S  L  R  *  R  S  I  L  R
+1:   S  V  P  V  I  R  H  L  F  A  P  L  K  E  Y  F  A

DNA: GTGTGTGTAAGGGACATGGGGGCAAACTGAGGTAGCG                  2995
+3:  V  C  V  R  D  M  G  A  N  *  G  S
+2:    V  C  K  G  H  G  G  K  L  R  *
+1:   C  V  *  G  T  W  G  Q  T  E  V  A
```

EXHIBIT C

LETTER

Estimation of Errors in "Raw" DNA Sequences: A Validation Study

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As DNA sequencing is performed more and more in a mass-production-like manner, efficient quality control measures become increasingly important for process control, but so also does the ability to compare different methods and projects. One of the fundamental quality measures in sequencing projects is the position-specific error probability at all bases in each individual sequence. Accurate prediction of base-specific error rates from "raw" sequence data would allow immediate quality control as well as benchmarking different methods and projects while avoiding the inefficiencies and time delays associated with resequencing and assessments after "finishing" a sequence. The program PHRED provides base-specific quality scores that are logarythmically related to error probabilities. This study assessed the accuracy of PHRED's error-rate prediction by analyzing sequencing projects from six different large-scale sequencing laboratories. All projects used four-color fluorescent sequencing, but the sequencing methods used varied widely between the different projects. The results indicate that the error-rate predictions such as those given by PHRED can be highly accurate for a large variety of different sequencing methods as well as over a wide range of sequence quality.

In DNA sequencing, knowledge about the accuracy of sequences can be very valuable. For example, different large-scale sequencing projects may produce sequences at similar rates and costs but with significantly different error rates in the final sequence. One major determinant in the final error rate is the accuracy of the "raw" sequence. Knowledge about the frequency and location of errors in the raw sequence data can help to direct "polishing" efforts to the places where additional effort is needed; it also enables the comparison between different sequencing projects without requiring that the same region be sequenced in each project.

Another area where estimates about sequence error rates would be beneficial is technology development. Accurate error estimates at each base would enable "quality benchmarking" between different methods, thus enabling researchers to choose the method that fills their needs for accuracy and throughput best.

Several groups have developed mathematical models to predict the error probability at any given position in raw sequences. Lawrence and Solovyev used linear discriminant analysis to calculate separate probability estimates for insertions, deletions, and mismatches (Lawrence and Solovyev 1994). Ewing and Green (1998) developed the program PHRED, which calculates a quality score at each base. This quality score q is logarithmically linked to the error probability p: $q = -10 \times \log_{10}(p)$ (for a discussion of how quality scores are calculated and what the limitations are, see Ewing et al. (1998). When used in combination with sequence assembly and finishing programs that utilize these error estimates, reliable error probabilities promise to increase the accuracy of consensus sequences and to reduce the efforts required in the finishing phase of sequencing projects (Churchill and Waterman 1992; Bonfield and Staden 1995).

To examine the accuracy of probability estimates made by the program PHRED, we compared the actual and predicted error rates for six different cosmid- or BAC-sized projects that were produced by six different large-scale sequencing centers in the United States. All of these six projects used four-color fluorescent sequencing machines; however, the DNA preparation methods, sequencing enzymes, fluorescent dyes and chemistries, and gel lengths varied significantly between the six groups. Table 1 gives an overview of the sequencing projects analyzed. Table 2 lists the different methods used.

RESULTS

Error Rate Prediction Accuracy for Six Projects

A comparison of actual and predicted error rates for the six projects in this study is shown in Table 3.

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Table 1. Summary of Data Sets

Project	Reads	Aligned bases	Average aligned read length
A	455	416,214	915
B	1277	871,230	682
C	1065	603,655	567
D	834	414,595	497
E	1638	1,149,209	702
F	1885	907,796	482
Total	7154	4,362,699	610

The results indicate that PHRED is very successful in identifying bases with low error probabilities. For example, the 1.28 million bases with quality scores of 4–12 (corresponding to error probabilities between 39.8% and 6.3%) contain a total of 187,926 errors. In contrast, the 1.44 million bases with quality scores between 33 and 42 (corresponding to error probabilities between 0.05% and 0.006%) contain only 237 errors, which translates into a 790-fold lower error rate. The trend toward lower error rates can also be observed for each individual project. In most cases, the actual number of errors is close to the predicted error rate. It is also apparent that the actual error rate is typically lower than the predicted error rate.

Both the high overall accuracy and the tendency to slightly overpredict errors are confirmed by statistical analysis, as shown in Table 4. The correlation between predicted and actual error frequencies is excellent for all projects (Spearman correlation coefficient >0.89, $P < 0.0001$). Averaged over all projects, the actual error rate is 84.5% of the predicted error rate; the slope of the relation between predicted and actual error rates differs slightly between projects and ranges from 76.6% to 88.4%. To put these differences between projects in relation, it is worthwhile remembering that PHRED quality scores cover a wide dynamic range: The maximum quality score of 51 corresponds to a 50,000-fold lower predicted error rate than the minimum quality score of 4. Even the relative difference between successive quality is larger than the relative difference in the slopes; for example, a quality score of 10 corresponds to an error probability of 10%, whereas a score of 9 corresponds to an error probability of 12.6%.

A different way of looking at the relation between the actual and predicted error rates is shown in Figure 1. Here, the error rates as a function of the position within all reads in each of the projects, averaged over 50-base windows, is depicted. For all six projects, the predicted error rates are very close to the actual error rates over the entire length of the sequences. Each project has a characteristic distribution of error rates, which differs from each of the other projects. The minimum error rate differs dramatically between projects. The best projects achieve raw error rates of 0.23%–0.36% in the best region of the sequence read, typically from base 150 to 200. The worst project in the data set had an ~10-fold higher error rate of 2.58%.

Toward the end of sequence reads, the error rates increase and start to exceed 10% between bases 300 and 700. In projects that used mainly short gels (e.g., projects D and F), this increase begins sooner, whereas projects that use longer gels show a markedly longer stretch of low error rates (e.g., projects A and B).

Table 5 summarizes key results for the six projects. The first four projects have similar minimum and average error rates. However, the length of the region where the error rate is below 5% differs significantly, from 403 to 682 bases. The project with the shorter low error rate regions contained larger portions of reads generated on short gels, whereas projects A and B were run exclusively on long gels (ABI373 stretch or ABI377 sequencers). Other factors contributing to differences between the first four projects were differences in sequencing chemistries, production scale, and electrophoresis conditions and machines.

Project E and, in particular, project F, had significantly higher error rates than the first four projects. In projects E and F, every sequence generated for the project had been included in the data set, whereas the other four projects had eliminated some "bad" sequences through manual or auto-

Table 2. Overview of Sequencing Methods Used in the Different Projects

Template DNA	single-stranded M13, double-stranded plasmids
Sequencing enzymes	Sequenase, *Taq*, KlenTaqTR, AmpliTaq FS
Sequencing chemistries	Dyes primer (two different dyes chemistries), dye terminator
Sequencing machines	ABI 373, ABI 373 stretch, ABI 377
Gel length	Only short gels, only long gels, mixes of short and long gels

Table 3. Comparison of Predicted and Actual Error Rates for Six Different Sequencing Projects

Project	Quality score	4–12	13–22	23–32	33–42	43–51
A	aligned bases	119,246	75,293	70,391	144,876	73,234
	expected errors	20,256	2,064	172	37	1
	actual errors	16,784	1,758	127	17	1
B	aligned bases	182,034	137,940	181,998	399,690	140,176
	expected errors	29,953	3,704	410	102	3
	actual errors	26,038	2,536	287	35	0
C	aligned bases	139,345	131,419	151,197	292,070	68,529
	expected errors	22,277	3,411	357	74	2
	actual errors	16,670	1,513	194	26	3
D	aligned bases	103,898	68,995	68,613	153,730	111,752
	expected errors	16,880	1,919	168	38	3
	actual errors	14,495	1,924	146	59	2
E	aligned bases	378,755	217,438	167,968	392,717	144,313
	expected errors	63,947	6,336	418	95	4
	actual errors	55,968	6,516	355	67	5
F	aligned bases	359,809	136,688	98,840	64,035	5,130
	expected errors	66,938	4,079	256	23	0
	actual errors	57,971	3,856	332	33	1
All	aligned bases	1,283,087	767,773	739,007	1,447,118	543,134
	expected errors	220,252	21,513	1,781	370	13
	actual errors	187,926	18,103	1,441	237	12

matic inspection. After eliminating <10% of the worst sequences in project F, the error rates for the remaining sequences were comparable to those of the first four projects. In contrast, project F showed a much more uniform distribution of sequence quality.

The last column in Table 5 shows the average number of bases with an estimated error probability of at most 0.1%, which is equivalent to a quality score of at least 30. The count of such "very high-quality" bases is a good indicator of sequence quality, both for individual sequences and, when aver-

Table 4. Summary of Statistical Analysis Results

Project	Spearman ρ	$P > \|\rho\|$	Slope	t ratio	$P > \|t\|$
A	0.9646	<0.0001	0.818	75.1	<0.0001
B	0.9890	<0.0001	0.874	98.2	<0.0001
C	0.9846	<0.0001	0.766	71.6	<0.0001
D[a]	0.8692	<0.0001	0.855	68.3	<0.0001
E	0.9956	<0.0001	0.884	144.3	<0.0001
F	0.9968	<0.0001	0.865	151.6	<0.0001
All	0.9964	<0.0001	0.845	174.5	<0.0001

[a]In project D, the Spearman correlation coefficient ρ was artificially low as only very few bases (10) bases had a quality score of 5, and none of these bases contained an actual error (expected: 3.16 errors). Exclusion of this quality score gave a Spearman correlation coefficient of 0.9786 ($P < 0.0001$). The frequencies in the slope calculations were weighed by the number of bases at any given quality score and, thus, were not sensitive to such small sample distortions (see Methods).

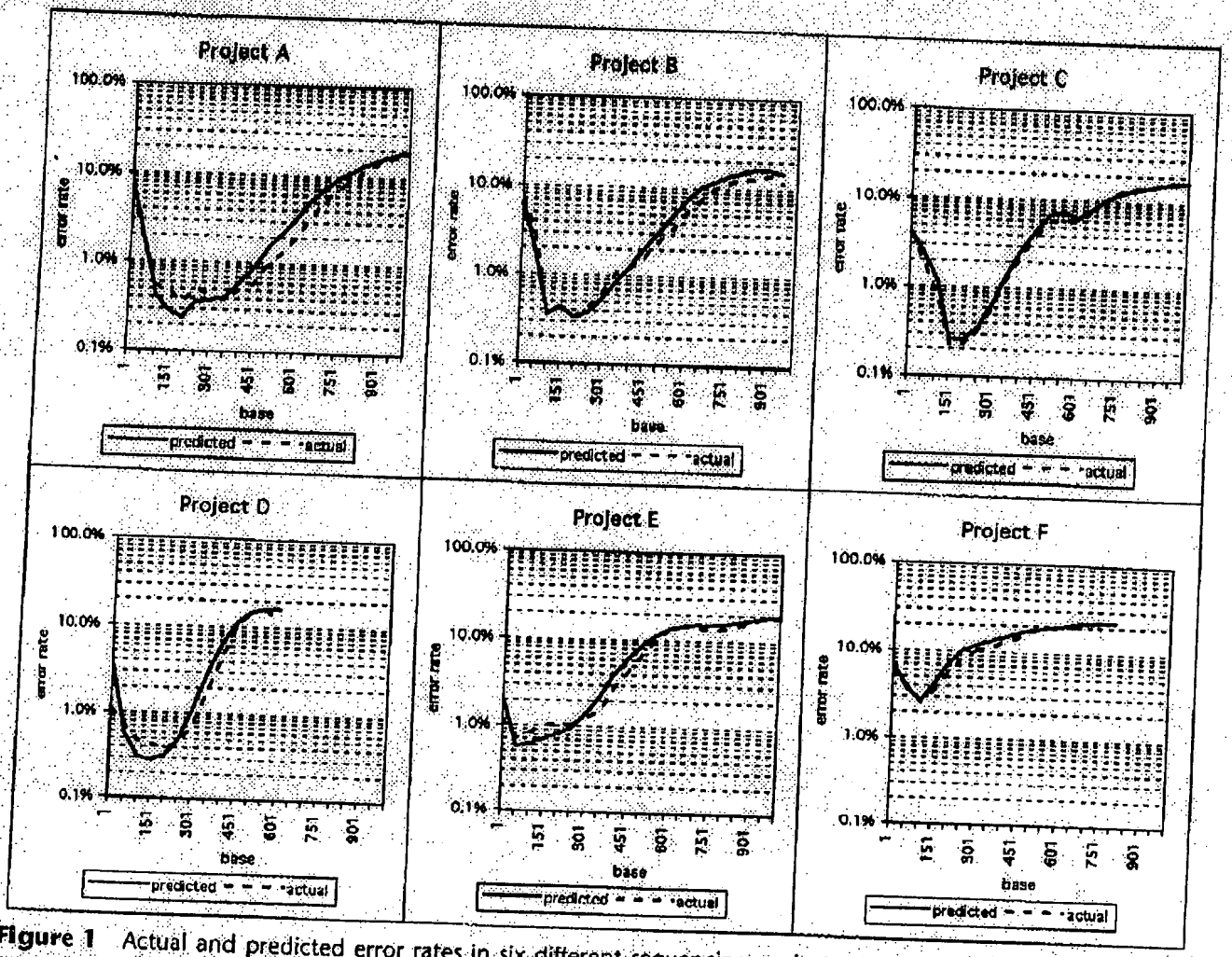

Figure 1 Actual and predicted error rates in six different sequencing projects. Actual error rates and predicted error rates in 50-base windows over the length of the sequence reads, averaged over all reads that could be aligned to the consensus sequence by CROSS_MATCH, are shown. The numbers on the *x*-axis show the first base in a given 50-base window.

aged over all sequences in a project, as an indicator for the entire project. Compared to the estimated error rates, the count of very high-quality bases is less prone to distortions from a small number of low-quality reads, as the data for project E demonstrate.

Prediction Accuracy for Data Subsets of Different Quality

The quality of sequences within any given project can vary substantially, and the use of predicted error rates has the potential to be a powerful tool for qual-

Table 5. Comparison of Key Results for Six Different Sequencing Projects

Project	Actual minimum error rate (%)	Actual average error rate (%)	Length of <1% error region	Length of <5% error region	Average bases with *P*(error) <0.1%
A	0.36	3.6	422	682	468
B	0.34	2.8	274	567	395
C	0.23	2.4	291	479	348
D	0.39	3.1	300	403	294
E	0.71	4.7	129	464	317
F	2.58	9.2	0	162	79

ity analysis and control in large-scale DNA sequencing projects. To analyze how accurate PHRED error estimates are for different quality sequences within the same sequencing project, we subdivided a data set into four quartiles, based on the number of very high-quality bases in each sequence (see Methods). The comparison of actual and predicted error rates is shown in Figure 2.

When measured by the error rate in the best region of a sequence, the data quality in the different quartiles varies >100-fold between the best and the worst 25% of the sequences. The best quartile showed ~0.03% error for >100 bases, whereas the error rate in the worst quartile always exceeded 5%. In quartiles 2 and 3, the predicted error rates match the actual error rates very closely. In the best and worst quartiles, PHRED's accuracy was somewhat lower from base 100 to 500. In the best sequences, PHRED's error estimates were about twofold too high; in the worst sequences, the error estimates were too low, again by a factor of 2. This underprediction of errors can be partially explained by the fact that PHRED gives ambiguous base calls (*N*'s) a quality score of 4, corresponding to an error probability of 39.8%; however, *N*'s will always show up as an actual error. Even in the worst and best quartiles, however, the predicted error rate curves are very similar to the actual error rate curves.

The results shown in Figure 2 also demonstrate that the count of very high-quality bases, or bases with an estimated error probability of at most 0.1%, can be used effectively to characterize the overall quality of a sequence read. Sorting the sequence reads into quartiles based on the number of very high-quality bases worked well, as shown by the >100-fold difference in the minimum error rate between the first and the fourth quartile.

Other methods to characterize the overall quality of individual reads based on PHRED quality scores can give similar results. For example, counting bases above a minimum quality threshold anywhere in the range of 20–40 gave similar results for most data sets (not shown), and such counts are used by a number of different laboratories as quality measures. Alternatively, the quality values can be converted to error probabilities and averaged to give the predicted error rate for the trace, or summed to give the total predicted number of errors in a trace. However, such averages and totals can sometimes give a misleading picture, as the following example illustrates. Assume that two sequence reads have very similar quality in the alignable part of the read but that one of the two sequences was run much longer and

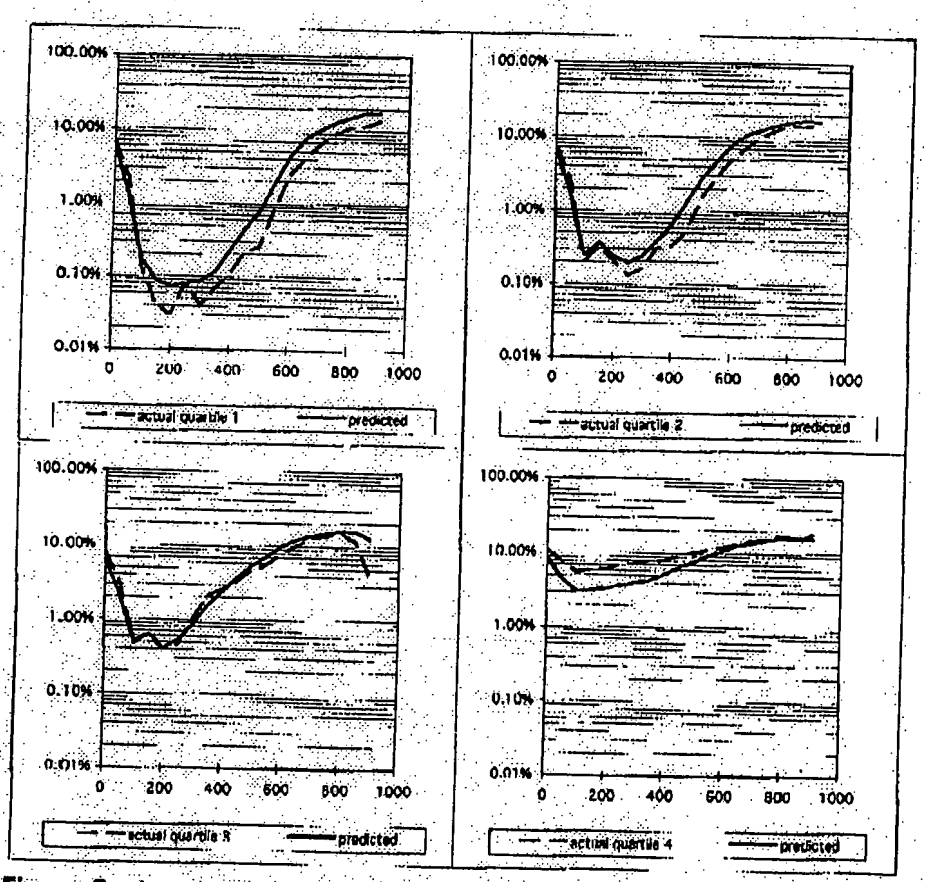

Figure 2 Actual and predicted error rates in different quality subsets of project B. Sequence reads were sorted by the number of bases with a predicted error rate of at most 0.1% (very high-quality bases), and assigned to quartiles, with quartile 1 corresponding to the highest numbers. Actual and predicted error rates for all sequences in each subset were calculated as in Fig. 1. Note that a number of sequence reads that had been rejected because of too low quality were added back to the data set for illustrative purposes, all of which are in quartile 4. These sequences were not included in the data sets used to generate Figs. 1 and 3 and Tables 1 and 3.

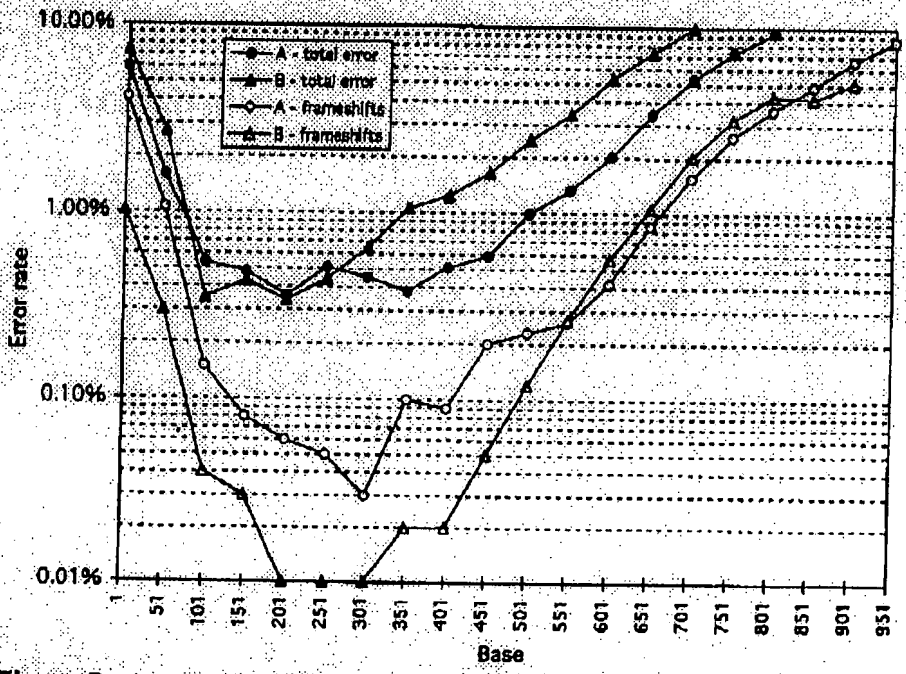

Figure 3 Actual frameshift and total error rates for projects A and B. To calculate frameshift error rates, only insertions and deletions were counted. Mismatch errors, which account for the vast majority of errors after base 150, were included only in the total error count. Note that project B (▲,△) has a slightly similar or slightly higher total error rate compared to project A (●,○) but only about one-third as many insertions and deletions up to base 500. For both projects, the frameshift error rate in the raw data is <1 in 1000 for >300 bases, and ≤1 in 10,000 for >100 bases in project B.

therefore contains a longer unalignable "tail" of very low-quality bases. When calculating the average error rate for these two sequences, the second sequence will have a much higher average error and, therefore, appear to be of lower quality. In contrast, the counts of very high-quality bases for both sequences will be very similar, as the unalignable tails contain few, if any, high-quality bases. Therefore, counts of bases above a high enough quality threshold will give a more robust and clearer picture of trace quality.

Frameshift Error Rates for Different Sequencing Chemistries

Depending on how biologists use DNA sequences, knowledge about total error rates in raw sequences may or may not be sufficient. For example, frameshift errors in coding sequences will generally lead to incorrectly predicted open reading frame, whereas mismatch errors will do so only if the mismatch introduces a stop codon or a new splice site. At the time of this writing, PHRED did not differentiate between mismatch and frameshift errors, but only estimated total error rates. This might occasionally lead to questionable conclusions, as the results shown in Figure 3 illustrate.

Figure 3 shows the total actual error rates and the frameshift error rates for two projects, A and B. The total error rates for both projects are similar for up to 350 bases; after 350 bases, project B has a somewhat higher total error rate. However, examining the frameshift error rate gives rise to a different picture: from base 1 to 500, project A has approximately four times as many insertions and deletions as project B. This difference in frameshift error rates can be explained by the sequencing chemistries that were used in the two projects. Project B, with the lower frameshift error rate, used only dye terminator chemistry, which is known to eliminate band spacing artifacts from hairpin structures ("compressions"). Project A, on the other hand, used dye primer chemistry, which is more prone to insertion and deletion errors from mobility artifacts, for most sequencing reactions.

DISCUSSION

As large-scale DNA sequencing has become a more routine and common process, the traditional methods for assessing sequence quality have become unsatisfactory. In projects like single-pass cDNA sequencing, it is not possible to calculate and compare error rates after finishing a sequence, as finishing never takes place. Even when a comparison between raw and finished sequence can be done, the time delay between raw data generation and quality assessment is often large. This delay makes it difficult to improve ongoing projects, and it sometimes makes it impossible to capture problems early on. Some immediate quality feedback can be reached by including known standard sequences for quality control. However, this approach can be costly, and it fails when error profiles differ between standard and unknown sequences.

In contrast to these traditional methods to assess sequence accuracy, direct estimation of error

rates in raw sequence data would enable immediate quality control and feedback. Accurate, base-by-base estimates of error probabilities could also increase the utility of single-pass sequences significantly, allow efficient comparison and optimization of different sequence chemistries, and enable the development of better software tools for sequence assembly and analysis.

The critical question for any error rate prediction tool is how accurate are the error rate estimates, in particular if different sequencing methods and chemistries are used? The results presented herein provide an answer to this question for the program PHRED, as well as clues where further development would be useful. As shown in Tables 3 and 4 and in Figure 1, the agreement between predicted and actual error rates was very good in each of the six different projects analyzed. The observed high level of prediction accuracy in all of these projects is almost astonishing if one takes into account that actual errors are binary (a base is either correct or wrong), whereas predicted error rates are probabilities on a scale from 0.0 to 1.0. The observed tendency to overpredict error rates can be at least partially explained by the "small sample correction" that was used in the derivation of threshold parameters for quality scores (Ewing and Green 1998). For most practical applications, such a somewhat conservative estimation of quality scores is tolerable or even desirable. Overall, the results clearly show that error probabilities given by PHRED accurately describe raw sequence data quality.

In judging the usefulness of predicted error probabilities, it is important to know how differences in sequencing methods will influence the prediction accuracy. For example, the larger variation in peak heights tends to be larger in dye terminator sequencing than in dye primer sequencing, and different sequencing enzymes are known to produce different specific height variation patterns. Any estimation of error probabilities that takes the peculiarities of a specific sequencing chemistry into account would therefore be expected to be less accurate for different chemistries.

The projects included in this study were specifically chosen to provide an initial answer to the question of how generally useful PHRED quality scores are. These projects represent the vast majority of different multicolor fluorescent sequencing methods used in the last 3 years: different template DNAs and DNA preparation methods, different enzymes, gel lengths, run conditions, and different fluorescent dyes. The data also include a considerable spread in data quality, both between projects and within individual projects. None of the projects analyzed here were included in PHRED's training set, and just one of the six laboratories that contributed data to this study also contributed data to the training data sets. One of the projects in this study consisted entirely of dye terminator sequences, which presented only a small fraction of the sequences in the test data set. Another project exclusively used a set of fluorescent dyes different from those used in the training sets. Each project differed from the other projects in this study in at least one, and typically many, experimental aspects like template preparation, sequencing enzymes, gel run conditions, and so forth. Despite these differences, the accuracy of error rate predictions was very similar for all projects.

Our results justify some optimism about the accuracy of PHRED quality scores for minor changes in sequencing technology, for example, sequences generated by new enzymes and fluorescent dyes. Initial studies showed that PHRED quality scores were also accurate for sequences produced by multiplex sequencing with radioactive detection (P. Richterich, unpubl.). However, we also observed two effects that can invalidate PHRED quality scores during these studies. First, sequences generated by chemical sequencing gave too low quality scores at mixed (A + G) reactions. Because secondary peak height is one of the parameters used in the error rate predictions, this is not surprising. Another potential source of error is high-frequency noise in the trace data. With such data, PHRED occasionally underestimated the band spacing by a factor of 2 or more, which resulted in incorrect base calls and quality scores. By applying simple smoothing algorithms to data with high-frequency noise, these problems could typically be resolved. Similar steps may be necessary to obtain accurate PHRED quality scores on data that have been generated by different sequencing instruments or preprocessed by different software.

Accurate quality scores can have a major impact on how sequences are used downstream from the sequence production process. In traditional sequencing projects where the goal is complete coverage at a final error rate below (e.g.) 1 in 10,000, the accuracy goals can be reached with single sequence reads as long as the quality scores are at least 40 (however, other potential problems like clone instability may make higher coverage advisable). Interesting questions arise as to how individual read quality contributes to project quality, or the error rate of the "final" sequence. Under the assumption that errors between different sequence reads are

completely independent, one could argue that two reads with a quality score of 20 (error probability of 1 in 100) are just as valuable as one sequence with a quality score of 40 (error probability of 1 in 10,000). However, although a single sequence stretch with quality levels above 40 would give a final sequence with an error rate of <1 in 10,000, assembling a consensus from two sequences with quality scores of 20 (1% error rate) could lead to one of two results: If the errors were completely random, the consensus sequence would be ambiguous at 2% of all locations; if the errors were completely localized, for example, because of reproducible compressions, the consensus sequence would have one "hidden" error every 100 bases. Typically, consensus sequences derived from low-quality sequences will have both kinds of problematic regions. Increased coverage can rapidly eliminate the random errors; however, increased coverage does not resolve errors from systematic sources. Manual examination of such problem areas is generally required; such "contig editing," however, tends to be time consuming, requires highly trained personnel, is an obstacle toward complete automation of DNA sequencing, and sometimes fails to eliminate all errors. This leads to the somewhat counterintuitive conclusion that the practical value of increasing sequence quality can be even higher than indicated by the quality scores: One sequence of average quality above 40 can be "worth" more than two sequences of average quality 20.

Another application of DNA sequencing where high quality can be of disproportionately high value is the search for mutations in genomic DNA. In low quality sequences, secondary peaks and low resolution often complicate the identification of heterozygous mutations. In regions of higher sequence quality, such secondary peaks are smaller or absent and peaks are better resolved. Therefore, both false-positive and false-negative errors can be significantly reduced in high-quality regions. Tools like PHRED, which can accurately measure sequence quality from trace data, can be of twofold value for mutation detection. First, base-specific quality scores can allow optimization of sequencing methods and strategies for mutation detection. Second, the quality scores can be used to evaluate the usefulness of individual sequence reads for mutation detection (e.g., by discarding reads below minimum thresholds), and they can guide software that automatically detects mutations.

The ability to predict error rates in a highly accurate fashion is likely to have a major impact in applications like those described above. PHRED is the first widely used program that accurately predicts base-specific error probabilities. However, the algorithm for determining quality values has been described (Ewing and Green 1998), and it should be straightforward to implement similar quality values in other base-calling programs. Furthermore, an extension of the approach developed by Ewing and Green should be possible. For example, differentiation between mismatch and frameshift errors would enable better comparisons of sequencing methods with similar total error rates but different frameshift error rates. Several groups have described efforts to calculate separate probabilities (or "confidence assessments") for mismatch errors and frameshift errors (Lawrence and Solovyev 1994; Berno 1996). Their results demonstrated that different approaches to error type characterization are feasible and promising. Implementation of such error type predictions in other programs similar to the way PHRED uses quality scores would enable better method assessments, benchmarking, and production quality control, and could have a significant impact on downstream uses of DNA sequence information.

METHODS

Data Sets

For one project, sequence raw data in the form of ABI trace files were downloaded from a public FTP site. Sequence data for the five other projects were kindly provided by five different large-scale sequencing groups. Table 1 gives a summary of the six projects, and Table 2 gives an overview of the different sequencing methods used in the projects. The projects differed in the amount of prescreening of data that had been done, reflecting different approaches to quality control in different laboratories. In two projects (B and C), different software programs had been used to identify and eliminate low-quality sequences. One project (F) included all data files generated, whereas the other three projects had excluded "failed lanes."

Comparison of Actual and Predicted Error Rates

The sequences for all traces in each project were recalled using the program PHRED (v. 961028). Next, sequences in each project were assembled with PHRAP (P. Green, unpubl.). Slightly different methods were chosen for the statistical and graphical evaluation of the error rate prediction accuracy. In the statistical evaluation, only the longest contig produced by PHRAP was considered. The tables of aligned bases and observed discrepancy counts for

each quality score were taken from the PHRAP output and analyzed as follows. The expected number of discrepancies (E) at each quality score (q) was calculated by multiplying the number of aligned bases (N) with the error probability corresponding to the quality score: $E = N\,10^{-q/10}$. The Spearman ranking coefficients were calculated by comparing the expected and observed error frequencies. To obtain the quantitative relation between the expected and observed error rates over the entire range, a least-squares fit between the observed and expected rates was performed, with the intercept set to zero and the number of aligned bases at each quality score used as weights.

For a graphical comparison of estimated and actual error rates in 50-bp windows, the following steps were taken. For two of the projects, the consensus sequence was retrieved from public databases. For the four other projects, the DNA sequence and quality information were used by the program PHRAP to assemble consensus sequences for each of the projects. The individual reads were aligned to the consensus sequences of the longest contig, using the program CROSS_MATCH (P. Green, unpubl.), after removing single-coverage regions from the ends of the consensus sequence. CROSS_MATCH uses an implementation of the Smith Waterman algorithm to generate alignments that typically do not include the ends of sequences, where disagreements are commonly due to vector sequence or low quality sequence.

The quality files generated by PHRED and the alignment summaries generated by CROSS_MATCH were then analyzed as follows. First, the region of each query sequence that had been aligned by CROSS_MATCH was determined. Next, the actual and predicted error rates for the entire aligned part of each individual sequence was calculated. In addition, the average actual and predicted error rates for all alignable sequences together were calculated for windows of 50 bases in length. To calculate the predicted error rate, the quality scores q determined by PHRED at each base were converted to error probabilities as described above (Ewing and Green 1998).

Subdividing Data Into Subsets Based on Data Quality

To examine the accuracy of PHRED quality scores for data subsets of different quality within a project, the following approach was taken. For all sequence reads in project B, the number of bases with a quality score of at least 30 in each sequence was determined (bases with quality scores of at least 30 were called very high-quality bases, or VHQ bases). Sequences were sorted in descending order based on the number of very high-quality bases, and divided into four quartiles. Accordingly, quartile 1 contained 25% of sequences with the highest number of very high-quality bases, and quartile 4 contained the "worst" sequences. To illustrate the prediction accuracy in data with relatively high error rates, sequences from project B that had been "discarded" because they had not met the minimum quality criteria were added back to the data set. The sequences in each quartile were compared to the consensus sequences that had been generated using the entire data set, as described above for the graphical comparison.

Determining Actual Frameshift Error Rates

The calculation of actual frameshift error rates in the raw sequence data was performed using CROSS_MATCH, similar to the procedure described above for total error rates, except that only insertion and deletion errors were counted. Because PHRED does not give separate frameshift error estimates, a comparison of predicted and actual frameshift errors is not possible.

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